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(54) Title: BACTERIAL MASS PRODUCTION OF TAXANES AND PACLITAXEL

(57) Abstract

The present invention relates to a method of obtaining different biologically pure cultures of bacteria isolated from different species of *Taxus* such as *Taxus canadensis, T. brevifolia, T. baccata, T. cuspi–data*, and *T. hunnewelliana*, wherein the bacteria produce *in vitro* taxanes and paclitaxel, and wherein the bacteria are of the genus *Sphingomonas, Bacillus, Pantoea* or *Curtobacterium*. Also, the present invention relates to a method of a bacterial mass production of at least one taxane or paclitaxel. There is also disclosed a novel bacterial taxane. The present invention also relates to the use of different biologically pure cultures of bacteria isolated from different species of *Taxus*, wherein the bacteria are able to biotransform pro–taxanes. There is also provided a process for improving taxanes and paclitaxel production of taxanes and paclitaxel producing bacteria which include culturing bacteria in the presence of a mutagenic agent for a period a time sufficient to allow mutagenesis. There is disclosed two new mutated bacterial which have an increased yield of pro–taxane biotransformation.

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BACTERIAL MASS PRODUCTION OF TAXANES AND PACLITAXEL

BACKGROUND OF THE INVENTION

(a) Field of the invention

The present invention relates to the production of paclitaxel and derivatives thereof (such as related taxanes) using a plurality of different bacteria isolated from different species of *Taxus*, and also to a novel taxane. There are disclosed methods for the isolation of these bacteria and the screening tests that were used to provide evidence that paclitaxel and taxanes were produced by said bacteria. There are also disclosed methods for the biotransformation of pro-taxanes by said bacteria.

(b) Description of prior art

Paclitaxel, also referred to as Taxol™, has been first identified in 1971 by Wani and collaborators (Wani MC *et al.*, 1971 *J. Am. Chem. Soc.*, **93**: 2325-2327) following a screening program of plant extracts of the National Cancer Institute. This complex diterpene showed cytotoxic activity against several types of tumors and is presently used in the treatment of some cancers such as ovarian and breast cancers. Clinical studies suggest that Taxol™ could eventually be used in the treatment of over 70% of human cancers.

Paclitaxel differs from other cytotoxic drugs by its unique mechanism of action. It interferes with cell division by manipulating the molecular regulation of the cell cycle. Paclitaxel binds to tubulin, the major structural component of microtubules that are present in all eukaryotic cells. Unlike other antimitotic agents such as vinca alkaloids and colcichine,

which inhibit the polymerization of tubulin, paclitaxel promotes this assembly of tubulin and stabilizes the resulting microtubules. This event leads to the interruption of cell division, and ultimately to cell death.

The major obstacle in the use of paclitaxel as an anticancer treatment is its supply. It was originally isolated from the bark and leaves of yew trees such as *Taxus brevifolia*, *T. baccata*, *T. cuspidata* or, *T. canadensis*. The low yield of the isolation of paclitaxel (0,016 g%) and the limited availability of the trees have forced the scientific and industrial community to find alternative ways of producing paclitaxel.

The antitumor property of taxoid compounds has also lead to the generation of new anticancer drugs derived from taxanes. Taxotere™ (sold by Rhône-Poulenc Rorer), which is produced from 10-deacetylbaccatin III by hemisynthesis, is currently used in the treatment of ovarian and breast cancers. Furthermore, Abbott Laboratories is conducting clinical trials with a drug derived from 9-dihydro-13-acetyl baccatin III, a natural precursor specific to *Taxus canadensis*. The increasing demand for taxanes highlights the urgent need for renewable and economical processes that would not endanger plant species.

Presently, industrials are producing paclitaxel through hemisynthesis from baccatin III, a natural precursor of paclitaxel . However, this process still relies on a plant substance that must be extracted from yew trees. The first complete chemical synthesis of paclitaxel has been achieved in 1994 by Nicolaou *et al.* (1994, *Nature*, **367**:630-634). This is a multistep proc-

ess and the overall yield has made this approach non economically feasible.

Plant cell culture of *Taxus* species is another approach explored by many groups (Yukimune *et al.*, 1996, *Nature Biotechnology*, **14**:1129-1132; Srinivasan *et al.*, 1995, *Biotechnology and Bioengineering*, **47**:666-676). Somehow, this process is limited by the amount of paclitaxel that can be produced, the length of incubation time required to obtain significant yields, and the application of plant cell culture to the large volumes required by the industry.

In United States Patent No. 5,322,779, in the names of Gary A. Strobel *et al.* disclosed a fungus isolated from the bark of a sample of *Taxus brevifolia* which is able to synthetize paclitaxel at a level of 24-50 ng/l after a period of incubation of 3 weeks. Later, Strobel *et al.* (1996, *Microbiology*, **142**:435-440) reported another fungus, *Pestalotiopsis microspora*, isolated from the inner bark of *Taxus wallachiana* that can produced up to 55 µg/l of paclitaxel within 5 weeks. Somehow, the long periods of incubation and the large volumes required to extract significant amounts of paclitaxel reduce the profitability of the process.

In United States Patent number 5,561,055 (issued on October 1, 1996 in the names of Michel Pagé *et al.*, the Applicant), there is disclosed one bacterium, which was referred to as *Erwinia taxi*, for the production of paclitaxel. Since then, this bacteria has been characterized as *Sphingomonas taxi*. This bacterium was isolated from *Taxus canadensis*. It would be highly desirable to be provided with other bacteria having

highly diverse metabolic capacities isolated from different species of *Taxus* for the production of paclitaxel and related taxanes at higher yields.

It would also be highly desirable to be provided with widely different bacteria for the mass production of various different bacterial taxanes.

As mentioned in International Patent Application published under number WO97/16200, biotransformation process may be used for the generation of new taxanes molecules that lead to new therapeutic drugs. It would also be highly desirable to be provided with new strains of microorganisms able to biotransform taxanes compounds for use as therapeutic agents or to be modified by hemisynthesis.

Genetic manipulations of bacteria can increase the activity or the production of certain proteins. It would also be highly desirable to be provided with mutant of our original isolates that could produce and biotransform taxanes at higher levels.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide a plurality of bacteria for the mass production of taxanes and paclitaxel.

Another aim of the present invention is to provide a method for bacterial mass production of taxanes and paclitaxel which overcomes all the drawbacks of the prior art.

Another aim of the present invention is to provide a novel process for the production of taxanes and paclitaxel. The industrial application of this process would provide alternative renewable sources of taxoids

compounds for the pharmaceutical industry.

Another aim of the present invention is to provide a biotransformation process in which plant-derived taxanes are converted into substances that may be useful for the production of other therapeutic compounds.

In accordance with the present invention there is provided a method to obtain biologically pure cultures of bacteria isolated from *Taxus*, wherein said bacteria produce *de novo* taxanes and paclitaxel at a concentration of about 1 to 25 µg/L, wherein said bacteria are isolated from the inner surfaces of different species of *Taxus* including without limitations *Taxus canadensis*, *T. brevifolia*, *T. hunnewelliana*, *T. baccata*, and *T. cuspidata*.

In addition, said bacteria are capable of producing biotransformed taxanes wherein pro-taxanes are added to their culture medium.

Such biologically pure cultures of bacteria of the present invention include, without limitation, bacteria of the genus selected from the group consisting of *Sphingomonas*, *Bacillus*, *Pantoea*, and *Curtobacterium*.

In accordance with the present invention, the bacteria include, without limitation, *Bacillus cereus* ssp. *taxi*, *Bacillus megaterium* ssp. *taxi*, *Pantoea* sp. BCM 1, *Pantoea* sp. BCM 2, *Pantoea* sp. BCM 3, *Bacillus cereus* ssp. BCM 4, *Bacillus subtilis* ssp. taxi, *Bacillus megaterium* ssp. BCM 9 or *Curtobacterium* sp. BCM 5.

In accordance with the present invention there is also provided a method of bacterial mass production of taxanes and paclitaxel thereof which comprises the steps of:

a) culturing the bacteria of the present invention in a growth-supporting nutrient medium capable of promoting growth and reproduction of said bacteria, and wherein said culturing is effected for a time sufficient to allow production of taxanes and paclitaxel; and

b) isolating said produced taxanes and paclitaxel from said bacteria or culturing medium of step a).

In accordance with the present invention there is also provided a process for improving taxanes and paclitaxel production of taxanes and paclitaxel producing bacteria comprising the steps of:

a) culturing bacteria in the presence of a mutagenic agent for a period of time sufficient to allow mutagenesis;

b) selecting said mutants by a change of the phenotype which results in an increased production of taxanes and paclitaxel.

The mutagenic agent may be a chemical agent, such as daunorubicin and nitrosoguanidine.

The mutagenic agent may be a physical agent, such as gamma radiation or ultraviolet.

The mutagenic agent may be a biological agent, such as a transposon.

In accordance with the present invention, the mutated bacteria include, without limitation, *Sphingomonas taxi* D200 or *Sphingomonas taxi* D201.

In accordance with the present invention there is also provided a method of bacterial biotransformation of taxanes and paclitaxel thereof which comprises the steps of:

a) incubating the bacteria of the present invention in a growth-supporting nutrient medium capable of promoting growth and reproduction of said bacteria, and wherein said incubation is effected in the presence of pro-taxanes for a time sufficient to allow production of taxanes and paclitaxel; and

b) isolating said produced taxanes and paclitaxel thereof from said culturing medium of step a).

In accordance with the present invention, there is also provided a process for improving biotransformation of pro-taxanes into taxanes and paclitaxel by taxanes and paclitaxel-producing bacteria comprising the steps of:

a) culturing bacteria in the presence of a mutagenic agent for a time sufficient to allow mutagenesis; and

b) selecting said mutants by a change of the phenotype which results in an increased biotransformation of pro-taxanes into taxanes and paclitaxel.

In accordance with the present invention there is also provided a method of bacterial biotransformation of pro-taxanes into taxanes and paclitaxel thereof which comprises the steps of:

a) incubating the mutated bacteria of the present invention in a nutrient medium, and wherein said incubation is effected in the presence of pro-taxanes for a time sufficient to allow production of taxanes and paclitaxel; and

b) isolating said produced taxanes and paclitaxel thereof from said culturing medium of step a).

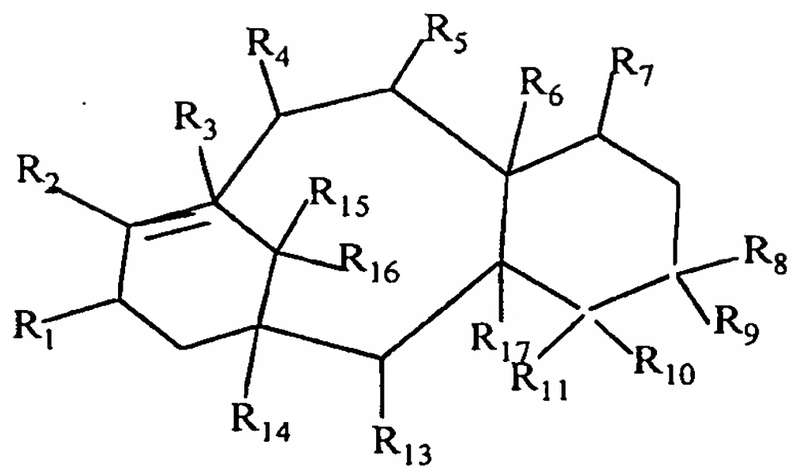
In accordance with the present invention there is also provided a novel bacterial taxane having a characteristic ultraviolet spectrum in HPLC as shown in Fig. 9B, Electrospray Ionization (EI) spectrum as shown in Fig. 10A, and characteristic fragments as shown in Fig. 10B.

This novel bacteria-derived taxane of the present invention is produced by at least two species of *Bacillus* (*B. cereus* ssp. *taxi* and *B. megaterium* ssp. *taxi*).

For the purpose of the present invention the following terms are defined below.

The term "taxanes and paclitaxel" is intended to mean any paclitaxel derivatives or precursor which have retained or not the taxol-associated cytotoxic biological activity or are thought to be precursors in the synthesis of paclitaxel. Such taxanes and paclitaxel may be selected from the group consisting of all the diterpenes isolated from any *Taxus* species. The production of all taxanes by bacteria, whether pharmacologically active or inactive, is contemplated within the scope of the present invention. Taxanes that are produced by the bacteria of the present invention may be as such found in *Taxus* plant species or may differs from the ones found in *Taxus* plant species.

Exemplary taxanes which may be produced by the bacteria of the present invention include but are not limited to those of the following Formula I:



I

wherein R_1, R_2, R_3, R_4, R_5, R_6, R_7, R_8, R_9, R_{10}, R_{11}, R_{12}, R_{13}, R_{14}, R_{15}, R_{16}, R_{17} are defined in Table 1 below.

However, since bacteria have highly diverse metabolic capacities the taxanes and paclitaxel must only correspond to Formula I, whatever is the nature of R_1 to R_{17} substituants.

The term "pro-taxanes" used in accordance with the present invention is intended to mean any precursors of any taxanes in the biosynthesis pathway of paclitaxel in plant, fungi and bacteria, including, without limitation, 10-deacetylbaccatin III, baccatin III, cephalomannine, taxinines, taxuspines, taxusin, 7-xylosyl taxol, 7-epi-10-deacetyl taxol, 10-deacetyl taxol, paclitaxel, 7-epitaxol, taxadiene, geranyl-geranyl-pyrophosphate (GGPP) and farnesyl-pyrophosphate.

Exemplary pro-taxanes which may be biotransformed by the bacteria of the present invention include but are not limited to those of the preceding formula I where R_1, R_2, R_3, R_4, R_5, R_6, R_7, R_8, R_9, R_{10}, R_{11}, R_{12}, R_{13}, R_{14}, R_{15}, R_{16}, R_{17} are defined in Table 1 below.

Exemplary biotransformed taxanes of the present invention include, but are not limited to, those of the

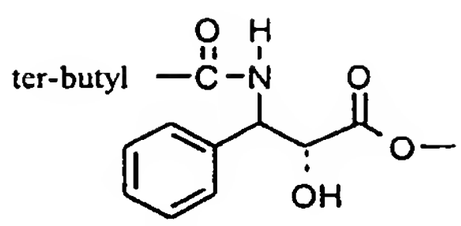
following Formula I where R_1, R_2, R_3, R_4, R_5, R_6, R_7, R_8, R_9, R_{10}, R_{11}, R_{12}, R_{13}, R_{14}, R_{15}, R_{16}, R_{17} are defined in Table 1.

Table 1

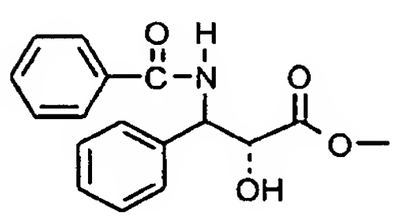
Compound	R_1[1]	R_2	R_3	R_4	R_5	R_6[3]	R_7[2]	R_8R_{17}	R_9R_{10}[4]	R_{11}	R_{12}[6]	R_{13}	R_{14}	R_{15}[5]	R_{16}
1) paclitaxel	tax	CH_3	H	β-acetyloxy	=O	β-CH_3	β-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
2) 10-deacetyl-cephalomannine	ceph	CH_3	H	β-OH	=O	β-CH_3	β-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
3) 7-epitaxol	tax	CH_3	H	β-acetyloxy	=O	β-CH_3	α-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
4) 10-deacetyl-7-epitaxol	tax	CH_3	H	β-OH	=O	β-CH_3	α-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
5) 7-epi-cephalomannine	ceph	CH_3	H	β-acetyloxy	=O	β-CH_3	α-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
6) baccatin III	α-OH	CH_3	H	β-acetyloxy	=O	β-CH_3	β-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
7) 10-deacetyl baccatin III	α-OH	CH_3	H	β-OH	=O	β-CH_3	β-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
8) cephalomannine	ceph	CH_3	H	β-acetyloxy	=O	β-CH_3	β-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
9) 10-deacetyl taxol	tax	CH_3	H	β-OH	=O	β-CH_3	β-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
10) 7-xylosyl taxol	tax	CH_3	H	β-acetyloxy	=O	β-CH_3	β-xylosyl	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
11) 7-xylosyl-cephalomannine	ceph	CH_3	H	β-acetyloxy	=O	β-CH_3	β-xylosyl	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
12) taxagifine	=O	α- CH_3	β-OH	β-acetyloxy	α-acetyloxy	β-CH_3	β-acetyloxy	H	α-cinnamoyloxy	methylene (=CH_2)	α-acetyloxy	β-H	H	cyclo	α- CH_3
13) δ-benzoyloxy-taxagifine	=O	α- CH_3	β-OH	β-acetyloxy	α-acetyloxy	β-benzoyl-oxymethyl	β-acetyloxy	H	α-cinnamoyloxy	methylene (=CH_2)	α-acetyloxy	β-H	H	cyclo	α- CH_3
14) 9-acetyloxy-taxusin	α-acetyloxy	CH_3	H	β-acetyloxy	α-acetyloxy	β-CH_3	H	H	α-acetyloxy	methylene (=CH_2)	H	H	H	CH_3	CH_3
15) 9-hydroxy-taxusin	α-acetyloxy	CH_3	H	β-acetyloxy	α-OH	β-CH_3	H	H	α-acetyloxy	methylene (=CH_2)	H	H	H	CH_3	CH_3
16) taxane Ia	tax	CH_3	H	=O	=O	β-CH_3	α-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
17) taxane Ib	taxsub	CH_3	H	=O	=O	β-CH_3	α-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
18) taxane Ic	taxsub	CH_3	H	=O	=O	β-CH_3	α-acetyloxy	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
19) taxane Id	α-acetyloxy	CH_3	H	β-acetyloxy	α-acetyloxy	β-CH_3	β-acetyloxy	H	α-OH	epoxide	α-acetyloxy	β-OH	H	CH_3	CH_3
20) 7-epibaccatin III	α-OH	CH_3	H	β-acetyloxy	=O	β-CH_3	α-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3

Footnotes

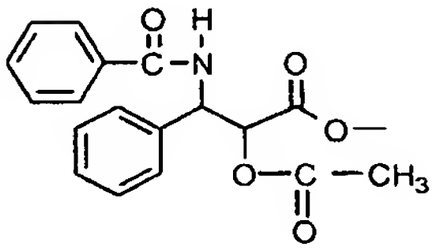
(1) "ceph" denotes



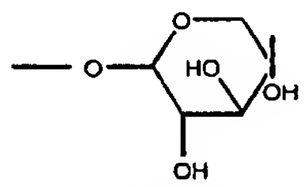
"tax" denotes



"taxsub" denotes



(2) "xylosyl" denotes



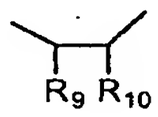
(3) "α" denotes the stereoposition of a stereomoiety below the plane of the taxane ring structure shown above

("⋯" ")

"β" denotes the stereoposition of a moiety above the plane of the taxane ring structure shown above

("▬" ")

(4) "oxetane" denotes the moiety



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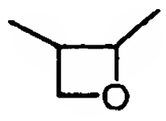
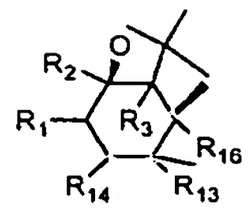


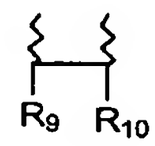
5) "cyclo" denotes the cyclic group formed by bonding the group

to the taxane

A ring as follows:



6) "epoxide" denotes the moiety



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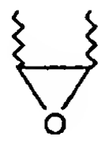

The term "taxol-associated cytotoxic biological activity" is intended to mean a cytotoxic activity which is sufficient to promote the assembly of tubulin and stabilizes the resulting microtubules of cancer cells causing the division of the cells in two equal daughter cells to be interrupted; and sufficient to cause a disruption in the dynamic equilibrium which exists between microtubules and their depolymerized tubulin dimers, thus preventing completion of the mitotic step which causes a lethal metaphase arrest of cancer cells.

The expression "cancer cells" is intended to mean any cancer cells which include without limitation, ovarian, breast, lung, head and neck cancer cells.

The term "growth supporting nutrient medium" is intended to mean any culture media which include, without limitation, carbon sources, nitrogen sources, amino acids, vitamins and minerals.

The term "intercalating agent" is intended to mean any molecule binding to the double stranded DNA structure which include, without limitation, daunorubicine, ethidium bromide, acridine orange, acriflavine and epirubicine.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A shows the elution profile, according to HPLC method no. 1, of an organic supernatant extract of a bacteria in accordance with one embodiment of the present invention, referred to as *Sphingomonas taxi*;

Fig. 1B illustrates the UV spectra of paclitaxel obtained from a standard and of an organic extract of supernatant of a bacteria in accordance with one embodiment of the present invention, referred to as *Sphingomonas taxi*;

Fig. 2A illustrates a typical chromatogram of an organic extract of the supernatant of a bacteria in accordance with one embodiment of the present invention, referred to as *Bacillus cereus* ssp. *taxi* using HPLC method no. 2;

Fig. 2B illustrates the ultraviolet spectrum of paclitaxel produced by *Bacillus cereus* ssp. *taxi* compared to a paclitaxel standard;

Fig. 2C illustrates the ultraviolet spectrum of 7-xylosyl-10-deacetyltaxol produced by *Bacillus cereus* ssp. *taxi* compared to an authentic standard co-eluting with taxinine M;

Fig. 3A illustrates a typical chromatogram of an organic extract of the supernatant of a bacteria in accordance with one embodiment of the present invention, referred to as *Pantoea* sp. BCM 1 using HPLC

method no. 2;

Fig. 3B illustrates the ultraviolet spectrum of 7-epi-10-deacetyltaxol produced by *Pantoea* sp. BCM 1 compared to an authentic standard;

Fig. 4A illustrates a typical chromatogram of an organic extract of the supernatant of a bacteria in accordance with one embodiment of the present invention, referred to as *Bacillus megaterium* ssp. *taxi* using HPLC method no. 2;

Fig. 4B illustrates the ultraviolet spectrum of the bacterial paclitaxel produced by *Bacillus megaterium* ssp. *taxi* compared to an authentic standard;

Fig. 5A illustrates a typical chromatogram of an organic extract of the supernatant of a bacteria in accordance with one embodiment of the present invention, referred to as *Bacillus cereus* ssp. BCM 4 using HPLC method no. 2;

Fig 5B shows a substance having the characteristic ultraviolet spectrum of a taxane compared to a standard of 7-xylosyl-10-deacetyltaxol co-eluting with taxinine M;

Fig. 6 illustrates the cytotoxicity of organic extracts of microorganisms isolated from different species of *Taxus* on CRL-1572 cell line as well as the cytotoxicity of taxanes and paclitaxel negative bacteria (STJ.BRP.K1 and *E. coli* TG1);

Fig. 7A illustrates the mass spectrometry of the substances eluted between 45 and 48 minutes using HPLC method no. 1 from an extract of *Sphingomonas taxi*;

Fig. 7B illustrates the mass spectrometry of a paclitaxel standard;

Fig. 8A illustrates the almost complete 16S rRNA

gene sequence of *Sphingomonas taxi* (SEQ ID NO:1);

Fig. 8B illustrates the almost complete 16S rRNA gene sequence of *Bacillus cereus* ssp. *taxi* (SEQ ID NO:2);

Fig. 8C illustrates the partial 16S rRNA gene sequence of *Bacillus megaterium* ssp. *taxi* (SEQ ID NO:3);

Fig. 8D illustrates the partial 16S rRNA gene sequence of *Pantoea* sp. BCM 1 (SEQ ID NO:4);

Fig. 8E illustrates the partial 16S rRNA gene sequence of *Bacillus cereus* ssp. BCM 4 (SEQ ID NO:5);

Fig. 8F illustrates the partial 16S rRNA gene sequence of a bacteria in accordance with one embodiment of the present invention, referred to as *Bacillus subtilis* ssp. taxi (SEQ ID NO:6);

Fig. 8G illustrates the partial 16S rRNA gene sequence of a bacteria in accordance with one embodiment of the present invention, referred to as *Pantoea* sp. BCM 2 (SEQ ID NO:7);

Fig. 8H illustrates the partial 16S rRNA gene sequence of a bacteria in accordance with one embodiment of the present invention, referred to as *Pantoea* sp. BCM 3 (SEQ ID NO:8);

Fig. 8I illustrates the partial 16S rRNA gene sequence of a bacteria in accordance with one embodiment of the present invention, referred to as *Bacillus megaterium* ssp. BCM 9 (SEQ ID NO:9);

Fig. 8J illustrates the partial 16S rRNA gene sequence of a bacteria in accordance with one embodiment of the present invention, referred to as *Curtobacterium* sp. BCM 5 (SEQ ID NO:10);

Fig. 8K illustrates the partial 16S rRNA gene

sequence of a bacteria in accordance with one embodiment of the present invention, referred to as *Sphingomonas* sp. BCM 7 (SEQ ID NO:11);

Fig. 9A illustrates a typical chromatogram of an organic extract from the supernatant of *Bacillus cereus* ssp. *taxi* producing a specific bacterial taxane using HPLC method no. 2;

Fig. 9B illustrates the ultraviolet spectrum of the characteristic taxane produced by *Bacillus cereus* ssp. taxi;

Fig. 10A illustrates the GC/MS (EI) spectrum of the purified specific bacterial taxane produced by *Bacillus cereus* ssp. *taxi* in accordance with the present invention;

Fig. 10B illustrates the mass-to-charge ratios of ions present in the EI mass spectra of the bacterial taxane produced by *Bacillus cereus* ssp. *taxi*;

Fig. 11A shows the evolution in time of two taxanes in the sterile culture medium supplemented with 1% v/v of an aqueous extract of *Taxus canadensis* incubated at 150 rpm at 30°C;

Fig. 11B shows the evolution of two taxanes transformed by *Sphingomonas taxi* when cultured in the culture medium S-7 supplemented with 1% of an aqueous extract of *Taxus canadensis*, shaken at 150 rpm at 30°C;

Fig. 12 shows the HPLC chromatograms of *S. taxi* cultured in the S-7 culture medium supplemented with 1% v/v of an aqueous extract of *Taxus canadensis* where sample name 8A represents the organic extract of the culture medium supplemented with 1% v/v of an aqueous extract of *Taxus canadensis*, sample name S 24 hres represents the organic extract of *Sphingomonas taxi* incu-

bated 24 hours in the culture medium supplemented with 1% v/v of an aqueous extract of *Taxus canadensis*, sample name S 48 hres represents the organic extract of *Sphingomonas taxi* incubated 48 hours in the culture medium supplemented with 1% v/v of an aqueous extract of *Taxus canadensis*, and sample name S 72 hres represents the organic extract of *Sphingomonas taxi* incubated 72 hours in the culture medium supplemented with 1% v/v of an aqueous extract of *Taxus canadensis*;

Fig. 13 compares HPLC chromatograms of the supernatant extracts of *S. taxi* (sample name: S 48 hres), *S. taxi D200* (sample name: SC 48 hres) and *S. Taxi D201* (sample name: SCP 48 hres) cultured 48 hours in the medium S-7 supplemented with 1% v/v of an aqueous extract of *Taxus canadensis*, and the organic extract of the medium S-7 supplemented with 1% v/v of an aqueous extract of *Taxus canadensis* (sample name 8A); and

Figs. 14A-14C show the characteristic ultraviolet spectrum of the new biotransformed pro-taxanes.

DETAILED DESCRIPTION OF THE INVENTION

Plants are hosts of a variety of microorganisms. The relation between the plant and the microorganism can be saprophytic, parasitic, endophytic or symbiotic. Whatever the relation, there may be genetic exchange between the species. *Taxus*, such as *Taxus canadensis*, which grows in some regions of the province of Quebec, shows significant amounts of paclitaxel in its needles and stems. Samples of *Taxus canadensis* from seven (7) regions of the province of Quebec were chosen as well as samples of different species of *Taxus* such as *Taxus*

brevifolia, *T. cuspidata*, *T. baccata*, *T. hunnewelliana*. Several different bacteria of different genus, such as *Sphingomonas*, *Bacillus*, *Pantoea*, and *Curtobacterium* were isolated from inner parts of samples from different species of Taxus, and all demonstrated taxanes and/or paclitaxel-producing properties.

Bacteria described above, produced taxanes and paclitaxel in fermentation procedures. Bacteria are cultured in a appropriate growth supporting nutrient medium containing ingredients known to those skilled in the art for the cultivation of microorganisms. Specific examples of appropriate such media are given below. Temperature of cultivation ranges from 10°C to 35°C, and aerobic cultivation is generally preferred.

Taxanes and paclitaxel are generally excreted in the supernatant, up to 10% of those substances remain in the cell. Taxanes and paclitaxel thereof may be extracted by performing an organic extraction with an appropriate solvents such as methylene chloride or ethyl acetate.

In accordance with the present invention, various bacteria producing taxanes and paclitaxel were isolated from different species of *Taxus*.

One bacterium isolated from *Taxus canadensis* allows for the production of taxanes and paclitaxel at a yield of 1 µg/L, referred to as *Sphingomonas taxi*, has been already deposited at the American Type Culture Collection but was identified as *Erwinia taxi* (ATCC, 12301 Parklawn Drive, Rockville, MD 20852 USA) on April 25, 1995 under deposit number ATCC 55669. The deposit is also available as required by Foreign Patent laws in

countries wherein counterpart applications are filed.

The other strains of the present invention have been deposited at the American Type Culture Collection (ATCC, 12301 Parklawn Drive, Rockville, MD 20852 USA) on December 18, 1997 under deposit accession numbers as set forth below. The deposit are also available as required by Foreign Patent laws in countries wherein counterpart applications are filed.

Strain	**ATCC No.**
Bacillus cereus ssp. taxi	202061
Bacillus megaterium ssp. taxi	202062
Curtobacterium sp. BCM5	202063
Pantoea sp. BCM2	202064
Bacillus megaterium BCM9	202065
Bacillus cereus BCM4	202066
Sphingomonas taxi D201	202067
Sphingomonas taxi D200	202068
Sphingomonas sp. BCM7	202069
Pantoea sp. BCM3	202070
Pantoea sp. BCM1	202071
Bacillus subtilis ssp. taxi	202072

In accordance with one embodiment of the present invention, the bacteria isolated from different species of *Taxus* allow for the production of taxanes and paclitaxel thereof at a yield of 1 to 25 µg/L.

In accordance with the present invention, the bacteria isolated from different species of *Taxus* may be employed for the biotransformation of pro-taxanes.

Isolation of the different microorganisms producing taxanes and paclitaxel

Each plant was divided into 5 parts; needles, twigs, stems, bark and roots. Each inner part of the plant was verified for the presence of taxanes and paclitaxel producing microorganisms.

The surface of every part of the plant was sterilized with 95% ethanol and then, cut into small pieces with a sterile blade. Pieces were homogenized in sterile water with a POLYTRON™ that had also been sterilized with ethanol 95%. The resulting mix was used to inoculate two different culture media; R2A agar (Difco) and Sabouraud agar (Difco).

Each plate was incubated at 22°C and examined on a day-to-day basis. The morphology of each colony was meticulously noted and the bacteria were transferred on different media until a pure culture was obtained. A Gram coloration of every bacteria was done before the culture was frozen at -80°C.

Over 50 bacteria were isolated from different samples of *Taxus canadensis* of the province of Quebec. In addition, over 30 different bacteria were isolated from different species of *Taxus* which include, without limitations, *Taxus brevifolia*, *T. baccata*, *T. cuspidata*, *T. hunnewelliana*. Some of them, showing taxanes and paclitaxel production capacities, will be fully described below.

Screening of microorganisms

In order to verify the production of taxanes and paclitaxel by microorganisms, each organism was cultured in at least 500 ml of a growth supporting nutrient medium. Any liquid medium allowing taxanes and

paclitaxel thereof production may be employed. Exemplary liquid media are S-7 media (Table 2), and defined media for *Bacillus* (Table 3). Every culture was performed in culture flasks and incubated at a temperature ranging from 20°C to 35°C with constant shaking until a sufficient growth was achieved, generally 18 to 72 hres.

Table 2

Composition of S-7 medium

Compounds	g/L
glucose	1
fructose	3
sucrose	6
sodium acetate	1
soytone	1
thiamine	0,001
biotine	0,001
pyridoxal-HCl	0,001
Ca pantothenate	0,001
$MgSO_4$	0,0036
$CaNO_3$	0,0065
$Cu(NO_3)_2$	0,001
$ZnSO_4$	0,0025
$MnCl_2$	0,005
$FeCl_3$	0,002
phenylalanine	0,005
sodium benzoate	0,1
KH_2PO_4 1M (pH 6,8)	1 ml

Table 3

Composition of the defined medium for *Bacillus*

Compounds	g/L
L-glutamic acid	10
glucose	5
citric acid	1
K_2HPO_4	0,5
KH_2PO_4	0,5
$MgSO_4-7H_2O$	0,2
$MnSO_4-4H_2O$	0,01
$FeSO_4-7H_2O$	0,01

The culture was then centrifuged and the pellet separated from the supernatant by decantation. To verify if taxanes and paclitaxel were secreted in the medium or if it was confined within the cells, both were tested for the presence of the drug. Since taxanes and paclitaxel are hydrophobic, and in order to concentrate each sample, an extraction with an organic solvent was performed. For the pellet, the cells were dried and about 200 mg were powdered and ultrasonicated twice for 40 minutes in 3 ml methanol. The extracts were dried at 25°C. The residue was dissolved by adding 2 ml of methylene chloride and 2ml of distilled water. After appropriate shaking, the mixture was centrifuged at 4 000 rpm for 5 min. The methylene chloride fraction was collected and dried under reduced pressure. Finally, the residue is dissolved in 0,5 ml of HPLC grade methanol.

The supernatant is extracted with one volume of methylene chloride. After appropriate shaking, the

organic fraction is evaporated to dryness under reduced pressure. The residue is then resolubilized in 50 ml of methylene chloride and 50 ml of distilled water. After appropriate shaking, each fraction was collected and dried under reduced pressure. Each residue is dissolved in a measured minimal volume of HPLC grade methanol. All samples were kept frozen at -20°C.

a) HPLC screening

HPLC method no. 1

Some extracts were analyzed by High Performance Liquid Chromatography (HPLC) on a system consisting of a WATERS™ 625 LC pump, a WATERS™ 996 photodiode array spectrophotometer, and a WATERS™ 717plus autosampler. Chromatography was performed with a phenyl column from Waters (5µm particle size, 6 mm X 15 mm) with a guard module. The injection volume varies from 50 to 150 µl and the flow rate maintained at 1 ml/min. The following elution program was used;

0 to 20 min.: methanol:water:acetonitrile (20:65:15) ramped to methanol:water:acetonitrile (20:45:35)

20 to 50 min.: methanol:water:acetonitrile (20:45:35) ramped to methanol:water:acetonitrile (20:25:55)

50 to 60 min.: methanol:water:acetonitrile (20:25:55) ramped to methanol 100%

Table 4 identifies the retention times of known authentic standards on HPLC methods no. 1 and no. 2. Using HPLC no. 1, paclitaxel has a retention time of 46 minutes. In Fig. 1, we show the ultraviolet spectrum

of paclitaxel produced by *Sphingomonas taxi*. The spectrum is very characteristic with a second maximum of absorption at 230nm. This figure illustrates that *Sphingomonas taxi* produces a compound having the same retention time and the same UV spectrum as paclitaxel.

HPLC method no. 2

Some extracts were analyzed on the same HPLC system with a curosil-PFP column (250 mm X 3.2 mm) from Phenomenex with a guard module. Injections varied from 50 ul to 150 ul and the flow rate maintained at 0.8 ml/min. The following gradient program was used;

0 to 50 min.: acetonitrile:water (25:75) ramped to acetonitrile:water (65:35)

50 to 62.5 min.: acetonitrile:water (65:35) ramped to methanol 100%

62.5 to 65 min.: methanol 100% to acetonitrile:water (25:75)

65 to 75 min.: acetonitrile:water (25:75)

As shown in Table 4, using HPLC method no. 2, paclitaxel is eluted at 36.987 minutes.

Table 4

Retention time of taxanes standards using HPLC methods no. 1 and no. 2

Taxanes	Retention time using	
	HPLC method no. 1	HPLC method no. 2
10-deacetyl baccatin III	n/a	12.037 min.
baccatin III	n/a	20.670 min.
7-xylosyl-10-deacetyltaxol B	n/a	24.870 min.
7-xylosyl-10-deacetyltaxol and taxinine M	n/a	27.120 min.
7-xylosyl-10-deacetyltaxol C	n/a	28.770 min.
10-deacetyltaxol and 7-xylosyltaxol	n/a	30.770 min.
cephalomannine	n/a	34.753 min.
7-epi-10-deacetyltaxol	n/a	35.703 min.
paclitaxel	46 minutes	36.987 min.
taxol C	n/a	38.853 min.
7-epitaxol	n/a	42.287 min.

Fig. 2A shows a typical chromatogram of an organic extract of *Bacillus cereus* ssp. *taxi*, and in Figs. 2B and 2C there is compared the UV spectra of the substances produced by *Bacillus* with authentic commercial plant standards. This figure clearly illustrates the ability of *Bacillus cereus* ssp. *taxi* to produce paclitaxel and 7-xylosyl-10-deacetyltaxol.

Fig. 3A shows the typical HPLC chromatogram of the supernatant of *Pantoea* sp. BCM 1 and, in Fig. 3B there is compared the bacterial 7-epi-10-deacetyltaxol against an authentic commercial plant standard, establishing the production of 7-epi-10-deacetyltaxol by *Pantoea* sp. BCM 1.

Fig. 4A shows a typical chromatogram of *Bacillus megaterium* ssp. *taxi* and, in Fig. 4B there is compared the ultraviolet spectrum of the bacterial paclitaxel

with an authentic standard proving the capacity of this bacterium to produce paclitaxel.

Fig. 5A shows a typical chromatogram of *Bacillus cereus* ssp. BCM 4 and, Fig 5B shows a substance having the characteristic ultraviolet spectrum of a taxane compared to a standard of 7-xylosyl-10-deacetyltaxol co-eluting with taxinine M, proving the capacity of *Bacillus cereus* ssp. BCM 4 to produce taxanes.

b) Cytotoxicity on cancer cells

An ovarian cancer cell line obtained from American Type Culture Collection (12301 Parklawn Drive, Rockville, MD 20852 USA) under ATCC accession number CRL-1572) was chosen for the investigation. Briefly, 2 000 cells/well of a 96-well microplate were inoculated. After 2 days, different dilutions of the drug were added in a volume of 100 µl. Three days later, the viability of cells was measured with ALAMAR™ blue (B. Pagé *et al.* 1993, *Intl. J. of Oncology*, 3:473-476). The ATCC CRL-1572 cell line is particularly sensitive to paclitaxel (ID_{50} of 1 ng/ml). Microbial extracts have also been tested for their cytotoxicity on those cells. Fig. 6 shows the cytotoxicity of different bacterial supernatant extracts. This figure clearly demonstrates that culture supernatant extracts from *Sphingomonas taxi* and *Bacillus cereus* ssp. *taxi* are at least 5-fold more cytotoxic than extracts from non-paclitaxel producing bacteria such as STJ.BRP.K1 and *E. coli* TG1.

c) Mass spectrometry

Mass spectrometry of the substances eluted between 45 and 48 minutes with HPLC method no.1 from an

extract of *Sphingomonas taxi* was performed. Fig. 7A shows the results obtained. In every sample, a substance with a molecular weight (M.W.) of 853,5 daltons appears. The theoretical M.W. of paclitaxel is 854 daltons. Even if these extracts were partially purified, essentially the major compound obtained is paclitaxel.

d) Characterization of a bacterial taxane by EI/MS

Fig. 9A, shows a typical chromatogram of *Bacillus cereus* ssp. *taxi* producing a characteristic taxane having a retention time of 15 minutes using HPLC method no. 2. In Fig. 9B, the characteristic ultraviolet spectrum of this substance is illustrated. It was purified on HPLC using HPLC method no. 2 and, analyzed in Electro Ionization Mass Spectrometry (EI/MS) as described by Thomas D. McClure et al. (*J. Am. Soc. Mass Spectrom.*, 1992, **3**, pp. 672-679).

Fig. 10A shows the EI spectrum of this taxane and Fig. 10B shows the characteristic fragments of this taxane. Since this taxane has never been observed in plant extracts of different species of *Taxus*, and this compound is produced by at least two species of *Bacillus* (*cereus* ssp. *taxi* and *megaterium* ssp. *taxi*), we consider this novel taxane unique to microorganisms isolated from *Taxus*.

Characterization of taxane and paclitaxel producing microorganisms

a) Biochemical and physiological characteristics

Table 5 shows the species of *Taxus* from which each bacterium was isolated, the Gram coloration, the

morphology of colonies on different culture media and, some biochemical characteristics. This Table clearly illustrates the diversity of our taxanes and paclitaxel producing bacteria i.e. we have isolated and identified Gram-negative and Gram-positive rods including Actinomycetes from different species of *Taxus* which all possess taxanes and/or paclitaxel producing capacities.

Table 5

Cellular, morphological and biochemical characteristics of some taxanes and paclitaxel producing bacteria

Name	Isolated from	Gram	Cellular morphology	Description of colonies on blood agar	Description of colonies on TA-1	Catalase	Urease
Sphingomonas taxi	*Taxus canadensis*	-	Rod	No growth	3 days to grow Orange-Opaque Glossy-Convexe Circular contour	+	-
Bacillus cereus ssp. taxi	*Taxus canadensis*	+	Rod	Double hemolysis Gray-green-Opaque Dry-Dull-Flat Irregular edge	Cream- Opaque Dry-Dull-Flat Irregular edge	+	-
Bacillus megaterium ssp. taxi	*Taxus hunnewelliana*	+	Rod	Gray- Opaque Dry-Dull Convex Irregular edge	Cream- Opaque Glossy Slightly convex Irregular edge	+	-
Pantoea sp. BCM 1	*Taxus hunnewelliana*	-	Rod	Semi-translucent Glossy-Flat Regular edge	Cream with yellow pigment in middle Opaque-Glossy-Flat Regular edge	+	-
Pantoea sp. BCM 2	*Taxus cuspidata*	-	Rod	Yellow Opaque Glossy-Flat Regular edge	Yellow Semi-translucent Glossy-Flat Regular edge	+	-
Pantoea sp. BCM 3	*Taxus cuspidata*	-	Rod	Semi-translucent Glossy-Flat Regular edge	Yellow Semi-translucent Glossy-Flat Regular edge	+	-
Bacillus cereus ssp. BCM 4	*Taxus brevifolia*	+	Rod	Double hemolysis Gray-green-Opaque Dry-Dull-Flat Irregular edge	Cream- Opaque Dry-Dull-Flat Irregular edge	+	-
Bacillus subtilis ssp. taxi	*Taxus baccata*	+	Rod	Semi-translucent Dry, Dull Convex Regular edge	Cream-Translucent Dry, Dull Convex Regular edge	+	-
Bacillus megaterium ssp. BCM 9	*Taxus hunnewelliana*	+	Rod	Gray- Opaque Dry-Dull Convex Irregular edge	Cream- Opaque Glossy Slightly convex Irregular edge	+	-
Curtobacterium sp. BCM 5	*Taxus brevifolia*	+	Rod	2 days to grow Cream Semi-translucent Glossy-Flat Regular edge	2 days to grow Yellow Semi-translucent Glossy-Flat Regular edge	+	-
Sphingomonas sp. BCM 7	*Taxus hunnewelliana*	-	Rod	No growth	3 days to grow Orange-Opaque Glossy-Convex Regular edge	+	-

b) Identification of the genus of taxanes and paclitaxel producing microorganisms

The genus of each taxanes and paclitaxel producing bacteria was determined by sequencing the 16S

rRNA genes. Genomic DNA of each strain was used as template for PCR (Polymerase Chain Reaction). Primers based on conserved regions at the beginning and the end of the 16S rRNA gene, SSU-27 (5'-AGAGTTTGATCMTGGCTCAG-3'; SEQ ID NO:12), and SSU-1 492 (5'-TACGGYTACCTTGTTACGACTT-3'; SEQ ID NO:13), were used to amplify a portion of the 16S gene. The amplicons were purified with the "PCR purification kit" (sold by Qiagen) and sequenced using the ABI Prism System. Sequence analysis was performed using GCG software package (Genetics Computer Group Inc., Madison, WI).

Fig. 8A shows the almost complete sequence of the 16S rRNA gene of *Sphingomonas taxi*. Since this strain has unique biosynthetic capacities and more than 3% sequence difference with the 16S rRNA genes of other known species of *Sphingomonas*, we created a new species and named it *taxi* on the behalf of its isolation source. Fig. 8B shows the almost complete sequence of the 16S rRNA gene of *Bacillus cereus* ssp. *taxi*. Since this bacterium possesses unique metabolic capacities, and in order to differentiate this species from other known *Bacillus cereus*, we identified it by subspecies name *taxi* also on the behalf of its isolation source. In Figs. 8C to 8L, we show partial sequences of the 16S rRNA genes of other taxanes and/or paclitaxel producing microorganisms.

Consequently, in accordance with the present invention, a plurality of bacteria isolated from different species of *Taxus* can be used for the mass production of paclitaxel and other taxanes. Based on the analysis of partial 16S rRNA gene sequences, and morphological and biochemical characteristics, we assigned

the following genera, species, and subspecies or strain names to our paclitaxel and taxanes producing bacterial isolates; *Sphingomonas taxi*, *Bacillus cereus* ssp. *taxi*, *Bacillus megaterium* ssp. *taxi*, *Pantoea* sp. BCM 1, *Pantoea* sp. BCM 2, *Pantoea* sp. BCM 3, *Bacillus cereus* ssp. BCM 4, *Bacillus subtilis* ssp. taxi, *Bacillus megaterium* ssp. BCM 9, *Curtobacterium* sp. BCM 5 and *Sphingomonas* sp. BCM 7.

Biotransformation of taxanes

a) **Preparation of an aqueous extract of *Taxus canadensis***

Fresh cuttings of needles and small twigs (10 g) of a sample of *Taxus canadensis* are homogenized in 100 ml of distilled water. The solution is then centrifuged at 7 000 rpm and the clear supernatant sterilized by filtration on a 0,22 µm filter. The solution is kept frozen at -20°C until utilization.

b) **Biotransformation of taxanes by taxanes and paclitaxel producing bacteria**

The growth supporting nutrient medium S-7 is supplemented with 1% v/v on an aqueous extract of *Taxus canadensis*. This resulting supplemented medium is then inoculated with a thawed vial of a pure culture of one of our strain and incubated at 30°C with constant shaking for a time sufficient to allow biotransformation of pro-taxanes.

The culture is then centrifuged and the remaining supernatant extracted with one volume of methylene chloride or ethyl acetate. After appropriate shaking, the organic fraction is evaporated to dryness under reduced pressure. The residue is then resolubilized in

50 ml of methylene chloride or ethyl acetate, and 50 ml of distilled water. After appropriate shaking, each fraction was collected and dried under reduced pressure. Each residues is dissolved in a measured minimal volume of HPLC grade methanol. All samples were kept frozen at -20°C until analysis.

Samples were analyzed on HPLC using method no. 2. Fig. 11 shows the evolution of two taxanes in A) the sterile culture medium S-7 supplemented with 1% v/v of an aqueous extract of *Taxus canadensis* shaken at 150 rpm and incubated at 30°C, and in B) the supernatant of *Sphingomonas taxi* cultured in the culture medium S-7 supplemented with 1% v/v of an aqueous extract of *Taxus canadensis* shaken at 150 rpm and incubated at 30°C. This figure clearly illustrates that in the supernatant of *Sphingomonas taxi*, the diminution of the taxane eluted at 12 minutes corresponds to the proportional elevation of the taxane eluted at 24 minutes, proving the capacity of *Sphingomonas taxi* to biotransform taxanes.

In addition, Fig. 12 compares the HPLC chromatogram of the organic extract of the culture supernatant of *S. taxi* incubated 24, 48 and 72 hours in the culture medium S-7 supplemented with 1% v/v of an aqueous extract of *Taxus canadensis*. This Fig. 12 clearly illustrates the production of pro-taxanes. The ultaviolet spectrum of one of these pro-taxanes is illustrated in Fig. 14A. Fig. 14A compares the UV spectrum of the new biotransformed taxane produced by *S. taxi* with taxanes from the aqueous extract of *Taxus canadensis*.

Mutagenesis of taxanes and paclitaxel producing bacteria

Typically, 20 ml of the culture medium TA-1 with 200 µg/ml of daunorubicin (purchased from Rhône-Poulenc) were inoculated with 500 µl of an overnight culture. The resulting broth was incubated at 200 rpm at 30°C for 2 days. After this time 10 ml of the broth are added to 10 ml of fresh medium containing 200 µg/ml of daunorubicin and incubated as described above. The preceding step is repeated as necessary to obtain mutated bacteria. Those mutants were further isolated on the solid culture medium TA-1 (composition as follows).

Solid culture medium TA-1

Ingredient	amount
glucose	5 g
tryptone	20 g
yeast extract	5 g
NaCl	0.5 g
agar	15 g
H_2O	1 L

Biotransformation of taxanes by mutated strains

The growth supporting nutrient medium S-7 is supplemented with 1% v/v on an aqueous extract of *Taxus canadensis*. This resulting supplemented medium is then inoculated with a thawed vial of a pure culture of one of our strain and incubated at 30°C with constant shaking for a time sufficient to allow biotransformation of pro-taxanes.

The culture is then centrifuged and the remaining supernatant extracted with one volume of methylene

chloride or ethyl acetate. After appropriate shaking, the organic fraction is evaporated to dryness under reduced pressure. The residue is then resolubilized in 50 ml of methylene chloride or ethyl acetate, and 50 ml of distilled water. After appropriate shaking, each fraction was collected and dried under reduced pressure. Each residues is dissolved in a measured minimal volume of HPLC grade methanol. All samples were kept frozen at -20°C until analysis.

Samples were analyzed on HPLC using method no. 2. Fig. 13 shows HPLC chromatograms of *S. taxi*, *S. taxi* D200 and *S. taxi* D201 incubated the same time (48 hours) in the culture medium S-7 supplemented with 1% v/v of an aqueous extract of *Taxus canadensis*. All cultures had comparable cell density. This figure clearly illustrates the improved yields of biotransformation by the mutated strains *S. taxi* D200 and *S. taxi* D201. In Figs. 14B and 14C the characteristic ultraviolet spectrum of the new pro-taxanes, produced by the mutated strains *S. taxi* D200 and *S. taxi* D201, are compared with the UV spectrum of two taxanes from *Taxus canadensis*.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather to limit its scope.

EXAMPLE I

Mass production of paclitaxel using *Sphingomonas taxi*

A colony of a pure culture of *Sphingomonas taxi* is used to inoculate 5 ml of S-7 culture medium. The broth is incubated 2-3 days with constant shaking (90 rpm) at 22°C. This 5 ml is then transferred into 5

liters of the same culture medium. The resulting broth is incubated as described above in aerobic conditions.

After 4-5 days of incubation, or after the maximum cell density is reached, the cell pellet is separated by centrifugation. Hydrophobic compounds are then extracted from the supernatant by partition with one volume of dichloromethane. Each organic fraction is evaporated to dryness and, the residue is solubilized in a minimal amount of HPLC grade methanol, typically 500 µl to 1 ml.

Paclitaxel and taxanes are further purified by HPLC on a phenyl column using HPLC method no. 1. Typically, up to 400 µl of the methanolic solution are injected and fractions of 0,5 ml to 1 ml are collected. Fractions containing paclitaxel or taxanes are evaporated to dryness.

Using this method, from 200 ng to 1 µg of paclitaxel per liter of culture medium were purified.

EXAMPLE II

Mass production of taxanes and paclitaxel using *Bacillus cereus* ssp. *taxi*

A thawed vial of a pure dense cell suspension of *Bacillus cereus* ssp. *taxi* is used to inoculate 500 ml of the defined medium for *Bacillus*. The broth is incubated 1 to 3 days with constant shaking (150 rpm) at 30°C. The cell pellet is then separated form the supernatant by centrifugation. Hydrophobic substances are extracted from the supernatant by an extraction with one volume of dichloromethane. The organic fraction is evaporated to dryness under reduced pressure,

and residues resolubilized in a minimal amount of HPLC grade methanol.

Taxanes and paclitaxel are further purified on HPLC using method no. 2. Using this method, from 0,2 to 10 µg of paclitaxel can be produced, and from 0,2 to 15 µg of taxanes, including the specific bacterial taxane illustrated in Fig. 13B, can also be produced.

EXAMPLE III

Mass production of taxanes and paclitaxel *using Bacillus megaterium* ssp. *taxi*

A thawed vial of a pure dense cell suspension of *Bacillus megaterium* ssp. *taxi* is used to inoculate 500 ml of the S-7 culture medium. The broth is incubated 1 to 3 days with constant shaking (150 rpm) at 30°C. The cell pellet is then separated form the supernatant by centrifugation. Hydrophobic substances are extracted from the supernatant by an extraction with one volume of dichloromethane. The organic fraction is evaporated to dryness under reduced pressure, and residues resolubilized in a minimal amount of HPLC grade methanol.

Taxanes and paclitaxel are further purified on HPLC using method no. 2. Using this method, from 1 to 12 µg of paclitaxel can be produced, and from 1 to 15 µg of taxanes, including the specific bacterial taxane illustrated in Fig. 13B, can also be produced.

EXAMPLE IV

Biotransformation of pro-taxanes by *Sphingomonas taxi*

The growth supporting nutrient medium S-7 is supplemented with 1% v/v on an aqueous extract of *Taxus*

canadensis. This resulting supplemented medium is then inoculated with a thawed vial of a pure culture of one of *Sphingomonas taxi* and incubated at 30°C with constant shaking for 24 to 96 hours.

The culture is then centrifuged and the remaining supernatant extracted with one volume of methylene chloride. After appropriate shaking, the organic fraction is evaporated to dryness under reduced pressure. The residue is then resolubilized in 50 ml of methylene chloride and 50 ml of distilled water. After appropriate shaking, the organic fraction is collected and dried under reduced pressure. The residue is dissolved in 500µl of HPLC grade methanol and 100 µl of the methanolic solution are analyzed on HPLC using method no. 2 and compared to the resulting chromatogram of the organic extract of the growth-supporting nutrient medium supplemented with and aqueous extract of *Taxus canadensis* 1% v/v shaken the same time.

As illustrated in Figs. 11, 12 and 14A, *Sphingomonas taxi* is able to biotransform taxanes into new pro-taxanes.

EXAMPLE V

Mutagenesis of *Sphingomonas taxi*

20 ml of the culture medium TA-1 with 200 µg/ml of daunorubicin (purchased from Rhône-Poulenc) were inoculated with 500 µl of an overnight culture of *Sphingomonas taxi*. The resulting broth was incubated at 200 rpm at 30°C for 2 days. After this time 10 ml of the broth are added to 10 ml of fresh medium containing 200 µg/ml of daunorubicin and incubated as described above. The preceding step is repeated as necessary to obtain mutated bacteria. Those mutants were further isolated on the solid culture medium TA-1. Two

new mutated strains were obtained named *Sphingomonas taxi* D200 and *Sphingomonas taxi* D201.

EXAMPLE VI

Biotransformation of taxanes by *Sphingomonas taxi* D200 and *Sphingomonas taxi* D201

The growth supporting nutrient medium S-7 is supplemented with 1% v/v on an aqueous extract of *Taxus canadensis*. This resulting supplemented medium is then inoculated with a thawed vial of a pure culture of one of our mutated strain and incubated at 30°C at 150 rpm. Cultures were stopped after 24, 48 and 72 hours of incubation.

Cultures were then centrifuged and the remaining supernatants extracted with one volume of ethyl acetate. After appropriate shaking, the organic fractions were evaporated to dryness under reduced pressure. Residues were then resolubilized in 50 ml of ethyl acetate. After appropriate shaking, fractions were collected and dried under reduced pressure. Each residues were dissolved in a measured minimal volume of HPLC grade methanol. All samples were kept frozen at -20°C until analysis.

Samples were analyzed on HPLC using method no. 2. Fig. 13 shows HPLC chromatograms of *S. taxi*, *S. taxi* D200 and *S. taxi* D201 incubated the same time (48 hours) in the culture medium S-7 supplemented with 1% v/v of an aqueous extract of *Taxus canadensis*. All cultures had comparable cell densities. This figure clearly illustrates the improved yields of biotransformation by the mutated strains *S. taxi* D200 and *S. taxi* D201. In Fig. 14B the characteristic ultraviolet spec-

trum of the new pro-taxanes produced by *S. taxi* D200 is compared with the UV spectrum of two taxanes from *Taxus canadensis*. Fig. 14C compares the UV spectrum of the new biotransformed taxane produce by *S. taxi* D201 with taxanes from the aqueous extract of *Taxus canadensis*.

While the invention has been described in connection with specific embodiments thereof, it will be understood that is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as many be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

American Type Culture Collection

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BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF
THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

BCM Developpement Inc.
Attn: Nathalie Landry
125 rue Dalhousie, suite 218
Quebec Canada G1K 4C5

REC'D 30 MAR 1999
WIPO PCT

Deposited on Behalf of: BCM Developpement

Identification Reference by Depositor:	**ATCC Designation**
Bacillus cereus **ssp. taxi**	**202061**
Bacillus megaterium **ssp. taxi**	**202062**
Curtobacterium **sp. BCM5**	**202063**
Pantoea **sp. BCM2**	**202064**
Bacillus megaterium **BCM9**	**202065**
Bacillus cereus **BCM4**	**202066**

The deposits were accompanied by: ___ a scientific description _X_ a proposed taxonomic description indicated above. The deposits were received December 12, 1997 by this International Depository Authority and have been accepted.

AT YOUR REQUEST: _X_ We will not inform you of requests for the strains.

The strains will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strains, and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strains.

If the cultures should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace them with living cultures of the same.

The strains will be maintained for a period of at least 30 years from date of deposit, or five years after the most recent request for a sample, whichever is longer. The United States and many other countries are signatory to the Budapest Treaty.

The viability of the cultures cited above was tested December 18, 1997. On that date, the cultures were viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC:

Barbara M. Hailey

Barbara M. Hailey, Administrator, Patent Depository

Date: December 18, 1997

cc: **France Cote**

American Type Culture Collection

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BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF
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RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT ISSUED PURSUANT TO RULE 7.3
AND VIABILITY STATEMENT ISSUED PURSUANT TO RULE 10.2

To: (Name and Address of Depositor or Attorney)

BCM Developpement Inc.
Attn: Nathalie Landry
125 rue Dalhousie, suite 218
Quebec Canada G1K 4C5

Deposited on Behalf of: BCM Developpement

Identification Reference by Depositor:	**ATCC Designation**
Sphingomonas **taxi D201**	**202067**
Sphingomonas **taxi D200**	**202068**
Sphinogomonas **sp. BCM7**	**202069**
Pantoea **sp. BCM3**	**202070**
Pantoea **sp. BCM1**	**202071**
Bacillus subtilis **ssp. taxi**	**202072**

The deposits were accompanied by: ___ a scientific description X a proposed taxonomic description indicated above. The deposits were received December 12, 1997 by this International Depository Authority and have been accepted.

AT YOUR REQUEST: X We will not inform you of requests for the strains.

The strains will be made available if a patent office signatory to the Budapest Treaty certifies one's right to receive, or if a U.S. Patent is issued citing the strains, and ATCC is instructed by the United States Patent & Trademark Office or the depositor to release said strains.

If the cultures should die or be destroyed during the effective term of the deposit, it shall be your responsibility to replace them with living cultures of the same.

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The viability of the cultures cited above was tested December 18, 1997. On that date, the cultures were viable.

International Depository Authority: American Type Culture Collection, Rockville, Md. 20852 USA

Signature of person having authority to represent ATCC:

Barbara M. Hailey

Barbara M. Hailey, Administrator, Patent Depository

Date: December 18, 1997

cc: **France Cote**

WHAT IS CLAIMED IS:

1. A process for producing a taxane, which comprises the steps of:

a) culturing at least one bacteria isolated from plant species of *Taxus* in growth-supporting nutrient medium capable of promoting growth and reproduction of said bacteria, and wherein said culturing is effected for a time sufficient to allow production of a taxane; and

b) recovering a taxane from said bacteria or medium of step a).

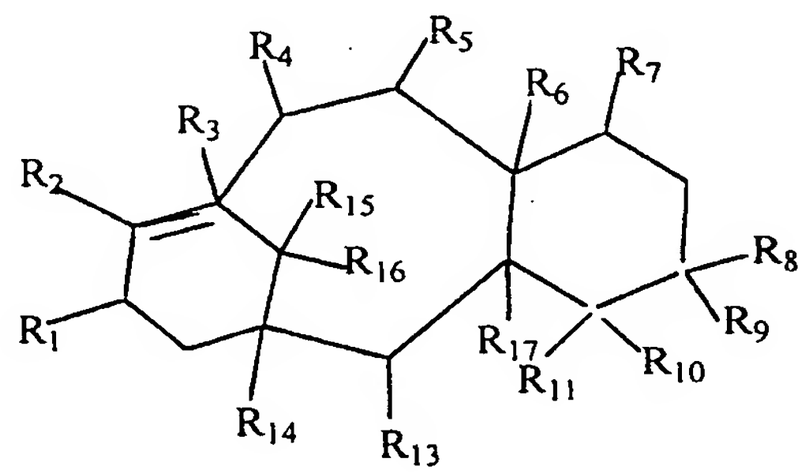
2. The process of claim 1, wherein the plant species of *Taxus* is of the species selected from the group consisting of but not limited to *Taxus canadensis*, *T. brevifolia*, *T. baccata*, *T. hunnewelliana* or *T. cuspidata*.

3. The process of claim 1, wherein said bacteria is of the genus selected from the group consisting of *Sphingomonas*, *Bacillus*, *Pantoea*, and *Curtobacterium*.

4. The process of claim 1, wherein said taxane produced is paclitaxel.

5. The process of claim 1, wherein said taxane is selected from the group consisting of paclitaxel, 10-deacetylcephalomannine, 7-epitaxol, 10-deacetyl-7-epitaxol, 7-epicephalomannine, baccatin III, 10-deacetyl-baccatin III, cephalomannine, 7-epibaccatin III, 7-

xylosyltaxol, 7-xylosyl-cephalomannine, taxagifine, δ-benzoyloxy taxagifine, 9-acetyloxy taxusin, 9-hydroxy taxusin, taxane Ia, taxane Ib, taxane Ic, taxane Id and any taxane corresponding to Formula I:



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wherein R_1, R_2, R_3, R_4, R_5, R_6, R_7, R_8, R_9, R_{10}, R_{11}, R_{12}, R_{13}, R_{14}, R_{15}, R_{16}, R_{17} are defined in Table 1.

SUBSTITUTE SHEET (RULE 26)

Table 1

Compound	R_1[1]	R_2	R_3	R_4	R_5	R_6[3]	R_7[2]	R_8R_{17}	R_9R_{10}[4]	R_{11}	R_{12}[6]	R_{13}	R_{14}	R_{15}[5]	R_{16}
1) paclitaxel	tax	CH_3	H	β-acetyloxy	=O	β-CH_3	β-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
2) 10-deacetyl-cephalomannine	ceph	CH_3	H	β-OH	=O	β-CH_3	β-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
3) 7-epitaxol	tax	CH_3	H	β-acetyloxy	=O	β-CH_3	α-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
4) 10-deacetyl-7-epitaxol	tax	CH_3	H	β-OH	=O	β-CH_3	α-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
5) 7-epi-cephalomannine	ceph	CH_3	H	β-acetyloxy	=O	β-CH_3	α-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
6) baccatin III	α-OH	CH_3	H	β-acetyloxy	=O	β-CH_3	β-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
7) 10-deacetyl baccatin III	α-OH	CH_3	H	β-OH	=O	β-CH_3	β-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
8) cephalomannine	ceph	CH_3	H	β-acetyloxy	=O	β-CH_3	β-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
9) 10-deacetyl taxol	tax	CH_3	H	β-OH	=O	β-CH_3	β-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
10) 7-xylosyl taxol	tax	CH_3	H	β-acetyloxy	=O	β-CH_3	β-xylosyl	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
11) 7-xylosyl-cephalomannine	ceph	CH_3	H	β-acetyloxy	=O	β-CH_3	β-xylosyl	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
12) taxagifine	=O	α- CH_3	β-OH	β-acetyloxy	α-acetyloxy	β-CH_3	β-acetyloxy	H	α-cinnamoyloxy	methylene (=CH_2)	α-acetyloxy	β-H	H	cyclo	α- CH_3
13) δ-benzoyloxy-taxagifine	=O	α- CH_3	β-OH	β-acetyloxy	α-acetyloxy	β-benzoyl-oxymethyl	β-acetyloxy	H	α-cinnamoyloxy	methylene (=CH_2)	α-acetyloxy	β-H	H	cyclo	α- CH_3
14) 9-acetyloxy-taxusin	α-acetyloxy	CH_3	H	β-acetyloxy	α-acetyloxy	β-CH_3	H	H	α-acetyloxy	methylene (=CH_2)	H	H	H	CH_3	CH_3
15) 9-hydroxy-taxusin	α-acetyloxy	CH_3	H	β-acetyloxy	α-OH	β-CH_3	H	H	α-acetyloxy	methylene (=CH_2)	H	H	H	CH_3	CH_3
16) taxane Ia	tax	CH_3	H	=O	=O	β-CH_3	α-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
17) taxane Ib	taxsub	CH_3	H	=O	=O	β-CH_3	α-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
18) taxane Ic	taxsub	CH_3	H	=O	=O	β-CH_3	α-acetyloxy	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
19) taxane Id	α-acetyloxy	CH_3	H	β-acetyloxy	α-acetyloxy	β-CH_3	β-acetyloxy	H	α-OH	epoxide	α-acetyloxy	β-OH	H	CH_3	CH_3
20) 7-epibaccatin III	α-OH	CH_3	H	β-acetyloxy	=O	β-CH_3	α-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3

6. A bacterial taxane having the characterizing ultraviolet spectrum and the specific retention time shown in Fig. 13, and the EI spectrum shown in Fig. 14A.

7. The process of claim 1, wherein the taxane produced having the characterizing ultraviolet spectrum and the specific retention time shown in Fig. 13, and the EI spectrum shown in Fig. 14A.

8. The process of claim 1, wherein the bacteria is *Bacillus cereus* ssp. *taxi*.

9. The process of claim 1, wherein the bacteria is *Bacillus megaterium* ssp. *taxi*.

10. The process of claim 1, wherein the bacteria is *Pantoea* sp. BCM 1.

11. The process of claim 1, wherein the bacteria is *Pantoea* sp. BCM 2.

12. The process of claim 1, wherein the bacteria is *Pantoea* sp. BCM 3.

13. The process of claim 1, wherein the bacteria is *Bacillus cereus* ssp. BCM 4.

14. The process of claim 1, wherein the bacteria is *Bacillus subtilis* ssp. *taxi*.

15. The process of claim 1, wherein the bacteria is *Bacillus megaterium* ssp. BCM 9.

16. The process of claim 1, wherein the bacteria is *Curtobacterium* sp. BCM 5.

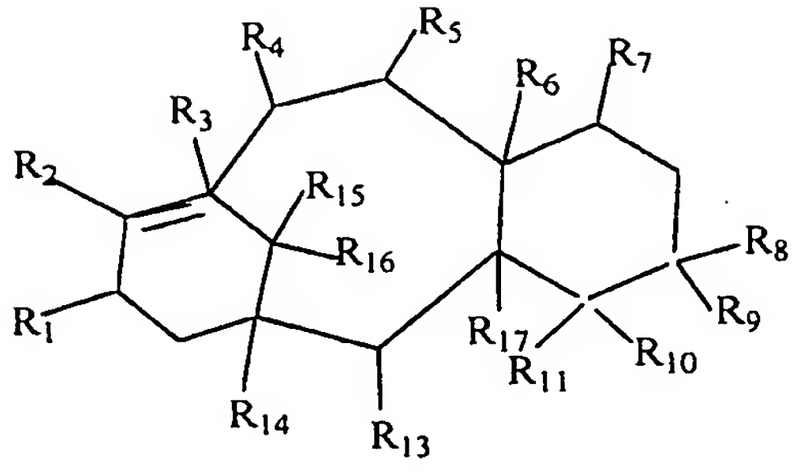
17. The process of claim 1, wherein said growth-supporting nutrient medium include carbon sources, nitrogen sources, amino acids, vitamins and minerals.

18. A process for producing biotransformed taxanes, which comprises the steps of:

a) culturing at least one bacteria isolated from plant species of *Taxus* in growth-supporting nutrient medium supplemented with pro-taxanes, and wherein said culturing is effected for a time sufficient to allow the biotransformation of pro-taxanes into taxanes; and

b) recovering at least one from said bacteria or medium of step a).

19. The process of claim 18, wherein said taxane is selected from the group consisting of paclitaxel, 10-deacetylcephalomannine, 7-epitaxol, 10-deacetyl-7-epitaxol, 7-epicephalomannine, baccatin III, 10-deacetylbaccatin III, cephalomannine, 7-epibaccatin III, 7-xylosyltaxol, 7-xylosyl-cephalomannine, taxagifine, δ-benzoyloxy taxagifine, 9-acetyloxy taxusin, 9-hydroxy taxusin, taxane Ia, taxane Ib, taxane Ic, taxane Id and any taxane corresponding to Formula I :



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wherein R_1 , R_2 , R_3 , R_4 , R_5 , R_6 , R_7 , R_8 , R_9 , R_{10} , R_{11} , R_{12} , R_{13} , R_{14} , R_{15} , R_{16}, R_{17} are defined in Table 1,

SUBSTITUTE SHEET (RULE 26)

Table 1

Compound	R_1[1]	R_2	R_3	R_4	R_5	R_6[3]	R_7[2]	R_8R_{17}	R_9R_{10}[4]	R_{11}	R_{12}[6]	R_{13}	R_{14}	R_{15}[5]	R_{16}
1) paclitaxel	tax	CH_3	H	β-acetyloxy	=O	β-CH_3	β-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
2) 10-deacetyl-cephalomannine	ceph	CH_3	H	β-OH	=O	β-CH_3	β-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
3) 7-epitaxol	tax	CH_3	H	β-acetyloxy	=O	β-CH_3	α-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
4) 10-deacetyl-7-epitaxol	tax	CH_3	H	β-OH	=O	β-CH_3	α-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
5) 7-epi-cephalomannine	ceph	CH_3	H	β-acetyloxy	=O	β-CH_3	α-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
6) baccatin III	α-OH	CH_3	H	β-acetyloxy	=O	β-CH_3	β-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
7) 10-deacetyl baccatin III	α-OH	CH_3	H	β-OH	=O	β-CH_3	β-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
8) cephalomannine	ceph	CH_3	H	β-acetyloxy	=O	β-CH_3	β-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
9) 10-deacetyl taxol	tax	CH_3	H	β-OH	=O	β-CH_3	β-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
10) 7-xylosyl taxol	tax	CH_3	H	β-acetyloxy	=O	β-CH_3	β-xylosyl	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
11) 7-xylosyl-cephalomannine	ceph	CH_3	H	β-acetyloxy	=O	β-CH_3	β-xylosyl	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
12) taxagifine	=O	α- CH_3	β-OH	β-acetyloxy	α-acetyloxy	β-CH_3	β-acetyloxy	H	α-cinnamoyloxy	methylene (=CH_2)	α-acetyloxy	β-H	H	cyclo	α- CH_3
13) δ-benzoyloxy-taxagifine	=O	α- CH_3	β-OH	β-acetyloxy	α-acetyloxy	β-benzoyl-oxymethyl	β-acetyloxy	H	α-cinnamoyloxy	methylene (=CH_2)	α-acetyloxy	β-H	H	cyclo	α- CH_3
14) 9-acetyloxy-taxusin	α-acetyloxy	CH_3	H	β-acetyloxy	α-acetyloxy	β-CH_3	H	H	α-acetyloxy	methylene (=CH_2)	H	H	H	CH_3	CH_3
15) 9-hydroxy-taxusin	α-acetyloxy	CH_3	H	β-acetyloxy	α-OH	β-CH_3	H	H	α-acetyloxy	methylene (=CH_2)	H	H	H	CH_3	CH_3
16) taxane Ia	tax	CH_3	H	=O	=O	β-CH_3	α-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
17) taxane Ib	taxsub	CH_3	H	=O	=O	β-CH_3	α-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
18) taxane Ic	taxsub	CH_3	H	=O	=O	β-CH_3	α-acetyloxy	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3
19) taxane Id	α-acetyloxy	CH_3	H	β-acetyloxy	α-acetyloxy	β-CH_3	β-acetyloxy	H	α-OH	epoxide	α-acetyloxy	β-OH	H	CH_3	CH_3
20) 7-epibaccatin III	α-OH	CH_3	H	β-acetyloxy	=O	β-CH_3	α-OH	H	oxetane	α-acetyloxy	α-benzoyloxy	β-OH	H	CH_3	CH_3

20. The process of claim 19, wherein said pro-taxanes are isolated from any species of *Taxus*.

21. The process of claim 19, wherein said pro-taxanes are isolated from *Taxus canadensis*.

22. The process of claim 19, wherein the bacteria is *Bacillus cereus* ssp. *taxi*.

23. The process of claim 19, wherein the bacteria is *Bacillus megaterium* ssp. *taxi*.

24. The process of claim 19, wherein the bacteria is *Pantoea* sp. BCM 1.

25. The process of claim 19, wherein the bacteria is *Pantoea* sp. BCM 2.

26. The process of claim 19, wherein the bacteria is *Pantoea* sp. BCM 3.

27. The process of claim 19, wherein the bacteria is *Bacillus cereus* ssp. BCM 4.

28. The process of claim 19, wherein the bacteria is *Bacillus subtilis* ssp. taxi.

29. The process of claim 19, wherein the bacteria is *Bacillus megaterium* ssp. BCM 9.

30. The process of claim 19, wherein the bacteria is *Curtobacterium* sp. BCM 5.

31. A process for isolating taxanes and paclitaxel producing bacteria from a yew tree, which comprises the steps of:

a) disinfecting the surface of a yew tree plant part with a suitable disinfectant;

b) cutting and disrupting the disinfected plant part;

c) leaching out bacteria with an appropriate solvent from the part of step c);

d) culturing said leached bacteria on a solid growth-supporting nutrient medium for a time sufficient to allow formation of visible isolated colonies; and

e) screening said isolated colonies for production of taxanes and paclitaxel.

32. The process of claim 31, wherein the disinfectant used is ethanol at concentration varying from 35% to 99%.

33. The process of claim 31, wherein the solvent to leach out bacteria is water.

34. The process of claim 31, wherein the solid growth-supporting nutrient medium for isolating colonies is selected from the group consisting of R2A agar (Difco) and Sabouraud (Difco).

35. The process of claim 31, wherein screening of isolated bacteria for taxanes and paclitaxel production comprises the steps of:

a) culturing said isolated bacteria in 500 ml of a growth-supporting liquid nutrient medium at temperature ranging from 20 to 35°C with constant shaking until sufficient growth is achieved;

b) obtaining the culture medium supernatant or the cells and extracting it with appropriate organic solvents selected from methylene chloride and ethyl acetate;

c) analyzing said organic extracts by a method suitable for detection of taxanes and paclitaxel.

36. The process of claim 35, wherein the method to detect taxanes and paclitaxel in extracts uses HPLC retention times on a hydrophobic chromatographic matrix and ultraviolet spectra to identify taxanes and paclitaxel in comparison with standards.

37. The process of claim 35, wherein the method to detect taxanes and paclitaxel in extracts uses cytotoxicity on cancer cell lines.

38. The process of claim 35, wherein the method to detect taxanes in extracts uses mass spectrometry to identify paclitaxel and taxanes based on molecular weights fragments and/or total molecule.

39. The process of claim 35, wherein the method to detect taxanes and paclitaxel in extracts uses antibodies raised against taxanes and paclitaxel.

40. The process of claim 35, wherein the method to

detect taxanes and paclitaxel uses an *in vitro* assay monitoring interaction of taxanes with tubulin.

41. A process for improving biotransformation of pro-taxanes into taxanes and paclitaxel by taxanes and paclitaxel-producing bacteria comprising the steps of:

a) culturing bacteria in the presence of a mutagenic agent for a time sufficient to allow mutagenesis; and

b) selecting said mutants by a change of the phenotype which results in an increased biotransformation of pro-taxanes into taxanes and paclitaxel.

42. The process of claim 41, wherein the mutagenic agent is a chemical agent.

43. The process of claim 41, wherein the chemical agent is daunorubicin or nitrosoguanidine.

44. The process of claim 41, wherein the mutagenic agent is a physical agent selected from gamma radiation or ultraviolet.

45. The process of claim 41, wherein the mutagenic agent is a biological agent.

46. The process of claim 45, wherein the biological agent is a transposon.

47. A method of bacterial biotransformation of pro-taxanes into taxanes and paclitaxel thereof which com-

prises the steps of:

a) incubating the mutated bacteria obtained according to the process of claim 41 in a nutrient medium, and wherein said incubation is effected in the presence of pro-taxanes for a time sufficient to allow production of taxanes and paclitaxel; and

b) isolating said produced taxanes and paclitaxel thereof from said culturing medium of step a).

48. The process of claim 43, wherein the bacteria used is *Sphingomonas taxi* D200.

49. The process of claim 43, wherein the bacteria used is *Sphingomonas taxi* D201.

50. The process of claim 43, wherein the mutated bacterium is isolated from a *Taxus* species and is of the genus of *Sphingomonas*, *Bacillus*, *Pantoea* or *Curtobacterium*.

51. A biologically pure culture of a bacteria of genus *Sphingomonas*, *Bacillus*, *Pantoea* or *Curtobacterium*, which is isolated from *Taxus canadensis*, and wherein said bacteria is characterized by the production of taxane and paclitaxel in a culture media.

52. The biologically pure culture of the bacteria of claim 51, which is *Sphingomonas taxi*, *Bacillus cereus* ssp. *taxi*, *Bacillus megaterium* ssp. *taxi*, *Pantoea* sp. BCM 1, *Pantoea* sp. BCM 2, *Pantoea* sp. BCM 3, *Bacillus cereus* ssp. BCM 4, *Bacillus subtilis* ssp. taxi, *Bacil-*

lus megaterium ssp. BCM 9 and *Curtobacterium* sp. BCM 5.

53. The biologically pure culture of the bacteria of claim 51, wherein the bacteria is of the genus *Sphingomonas*, belonging to the alpha subdivision of Proteobacteria.

54. The biologically pure culture of the bacteria of claim 51, wherein the bacteria is of the genus *Bacillus* belonging to the low G+C Gram positive bacteria.

55. The biologically pure culture of the bacteria of claim 51, wherein the bacteria is of the genus *Pantoea*, belonging to the gamma subdivision of Proteobacteria.

56. The biologically pure culture of the bacteria of claim 51, wherein the bacteria is of the genus is isolated from a *Taxus* species and is of the genus *Curtobacterium*, belonging to the Actinomycetes order of the firmicutes.

57. The biologically pure culture of the bacteria of claim 51, wherein said bacteria is mutated and is *Sphingomonas taxi* D200 or *Sphingomonas taxi* D201.

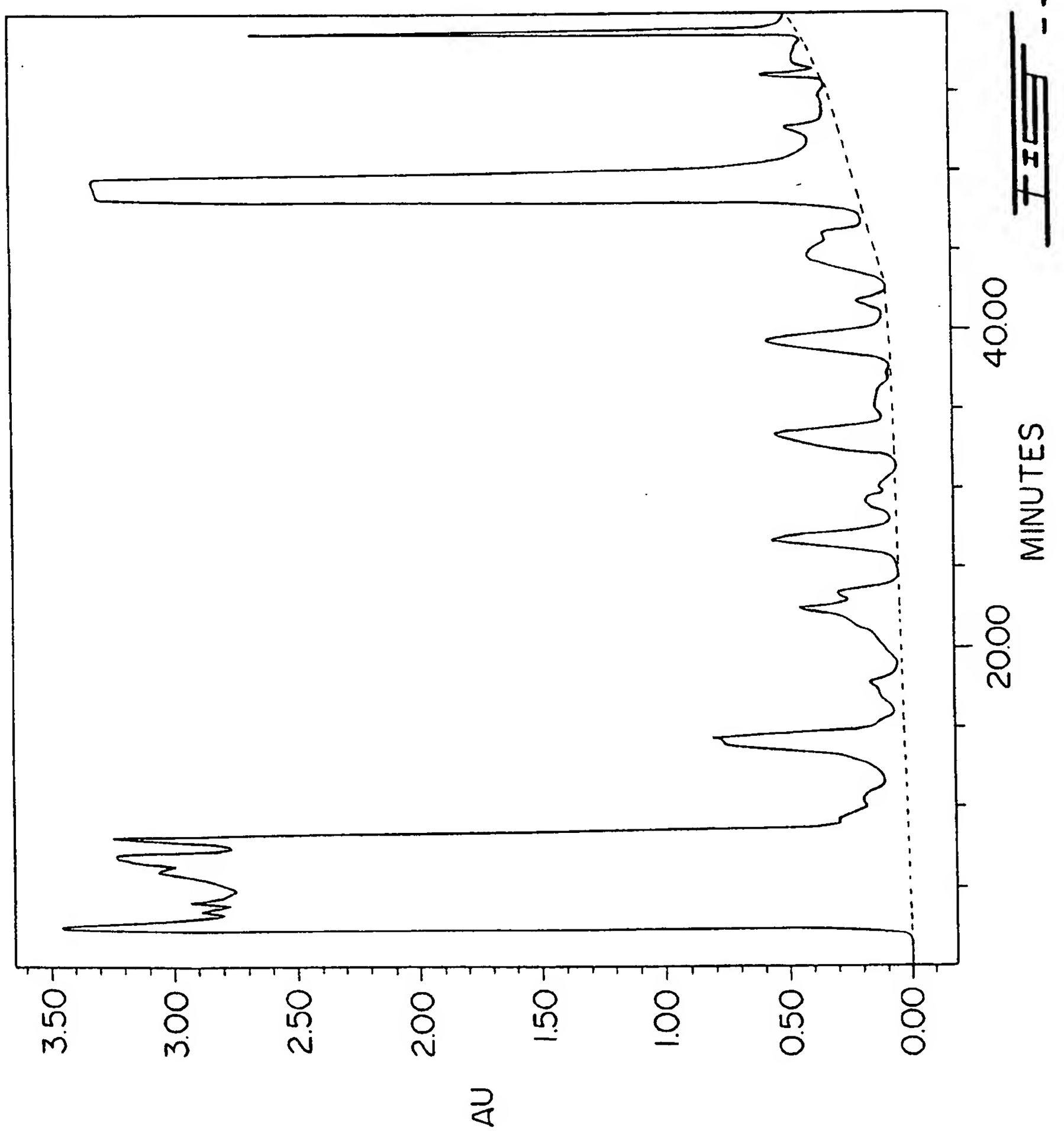


FIG - 1A

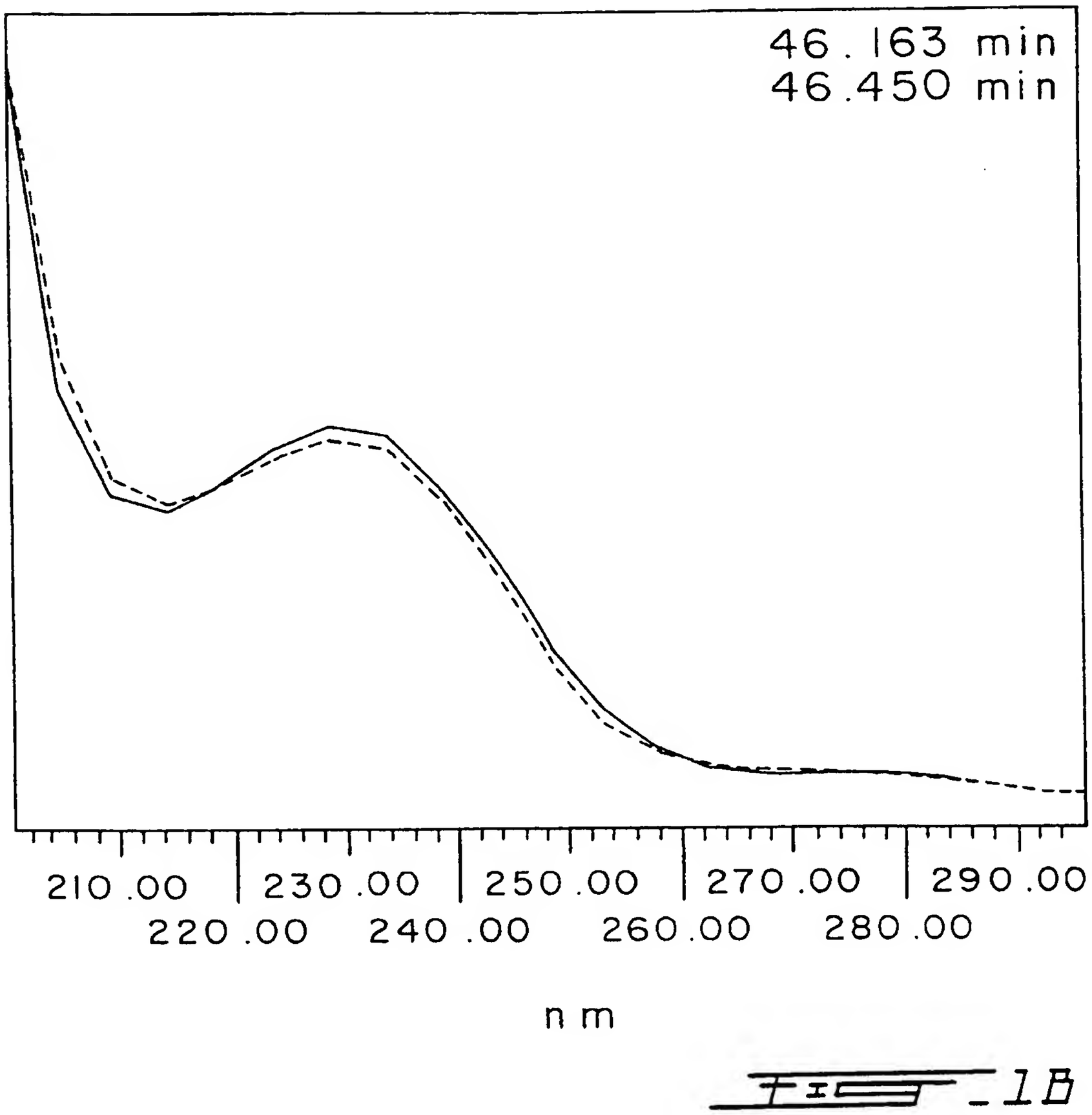

46.163 min
46.450 min
210.00
220.00
230.00
240.00
250.00
260.00
270.00
280.00
290.00
nm

Fig 1B

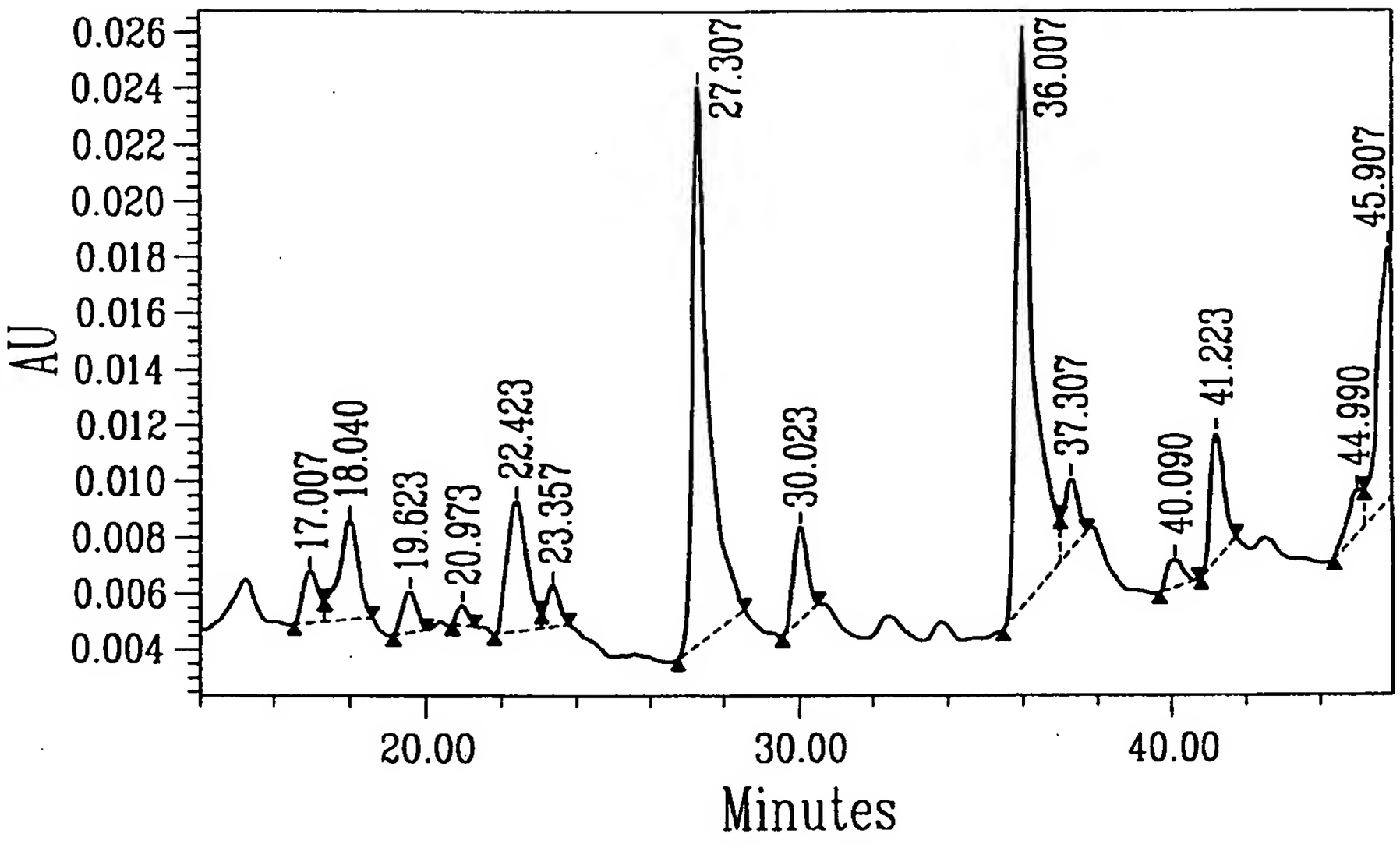

0.026
0.024
0.022
0.020
0.018
0.016
0.014
0.012
0.010
0.008
0.006
0.004
AU
17.007
18.040
19.623
20.973
22.423
23.357
27.307
30.023
36.007
37.307
40.090
41.223
44.990
45.907
20.00
30.00
40.00
Minutes

FIG 2A

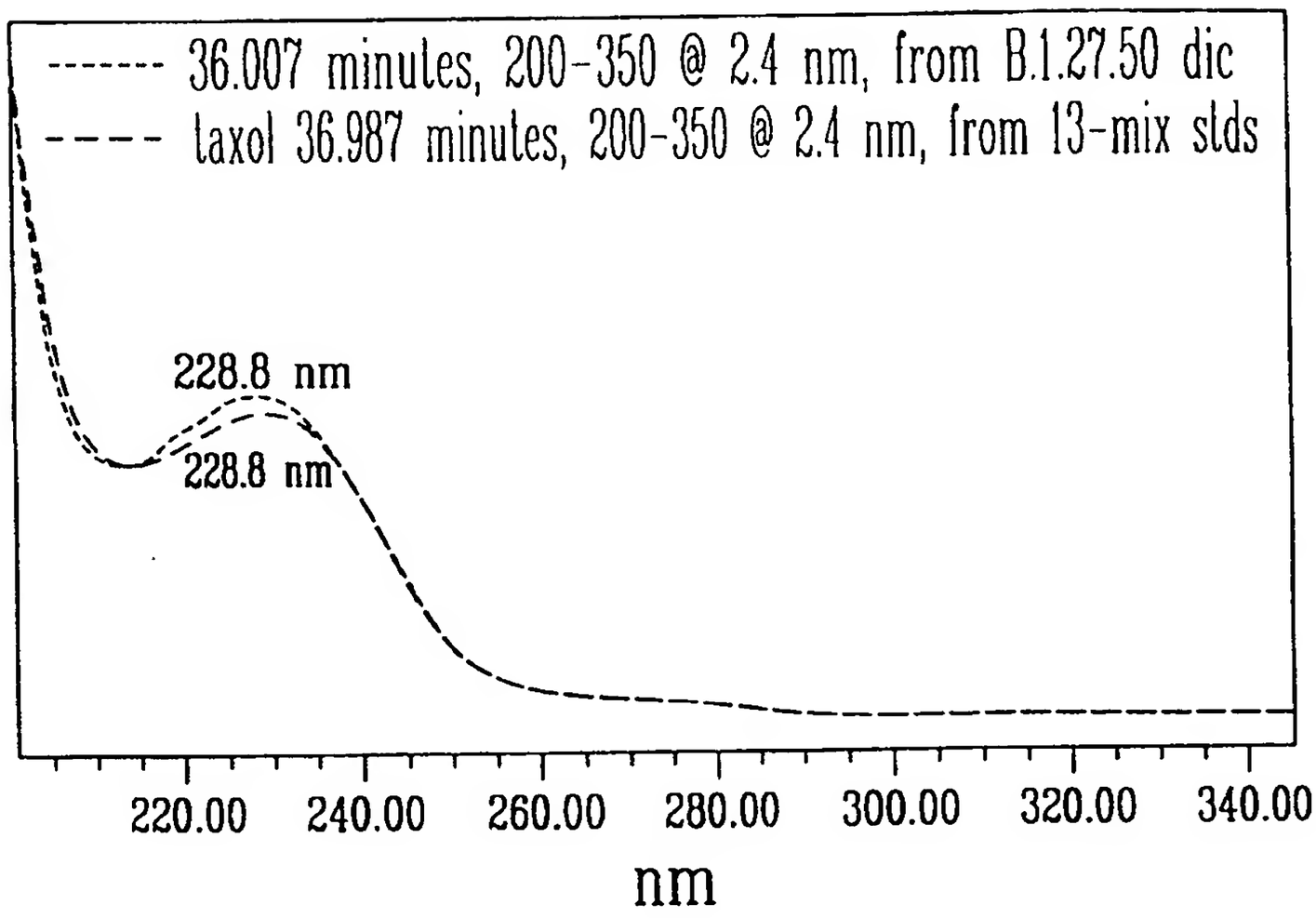

------ 36.007 minutes, 200-350 @ 2.4 nm, from B.1.27.50 dic
---- taxol 36.987 minutes, 200-350 @ 2.4 nm, from 13-mix stds
228.8 nm
228.8 nm
220.00
240.00
260.00
280.00
300.00
320.00
340.00
nm

FIG - 2B

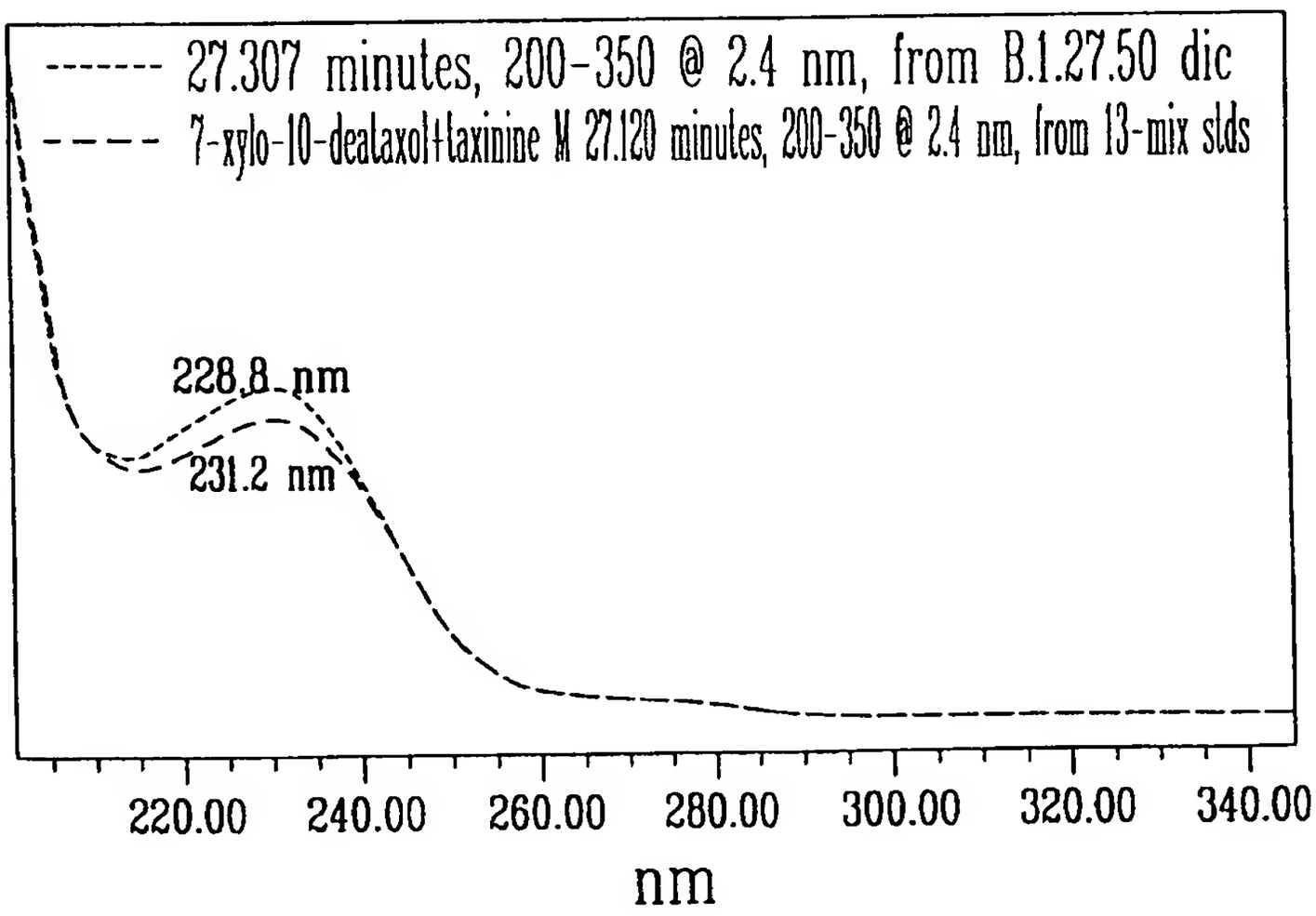

------ 27.307 minutes, 200-350 @ 2.4 nm, from B.1.27.50 dic
---- 7-xylo-10-deataxol+taxinine M 27.120 minutes, 200-350 @ 2.4 nm, from 13-mix stds
228.8 nm
231.2 nm
220.00
240.00
260.00
280.00
300.00
320.00
340.00
nm

FIG - 2C

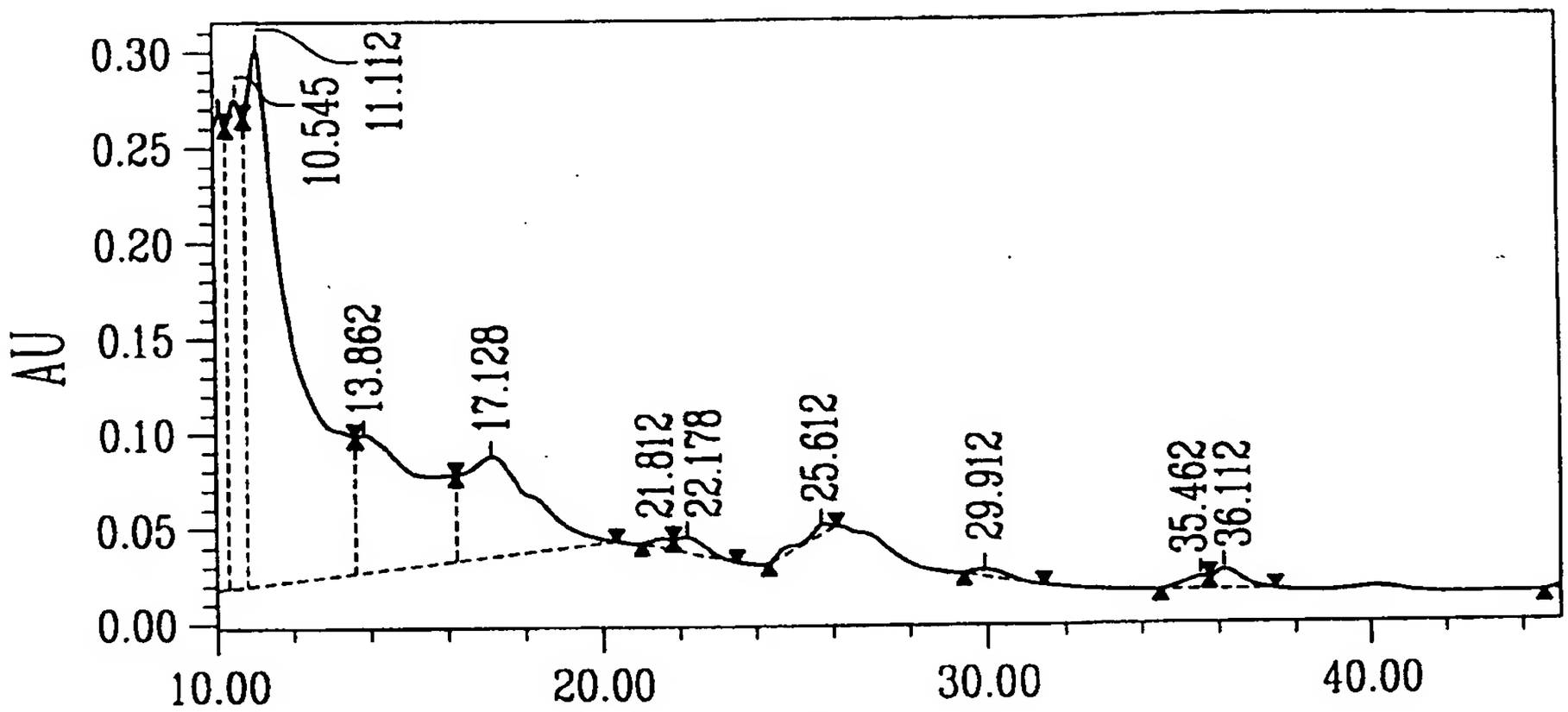

AU
0.30
0.25
0.20
0.15
0.10
0.05
0.00
10.545
11.112
13.862
17.128
21.812
22.178
25.612
29.912
35.462
36.112
10.00
20.00
30.00
40.00
Minutes

FIG - 3A

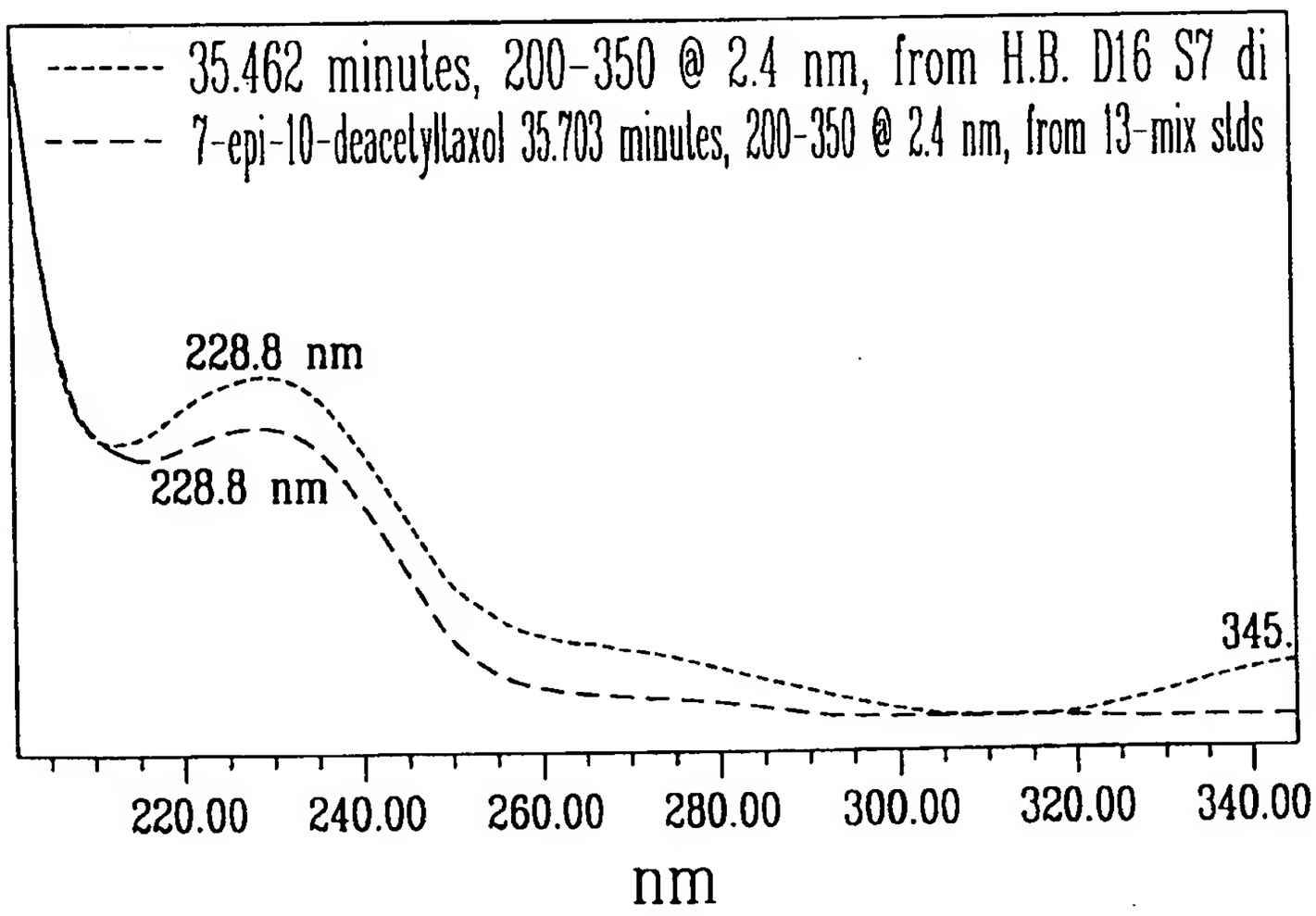

------ 35.462 minutes, 200-350 @ 2.4 nm, from H.B. D16 S7 di
- - - - 7-epi-10-deacetyltaxol 35.703 minutes, 200-350 @ 2.4 nm, from 13-mix stds
228.8 nm
228.8 nm
345.
220.00
240.00
260.00
280.00
300.00
320.00
340.00
nm

FIG - 3B

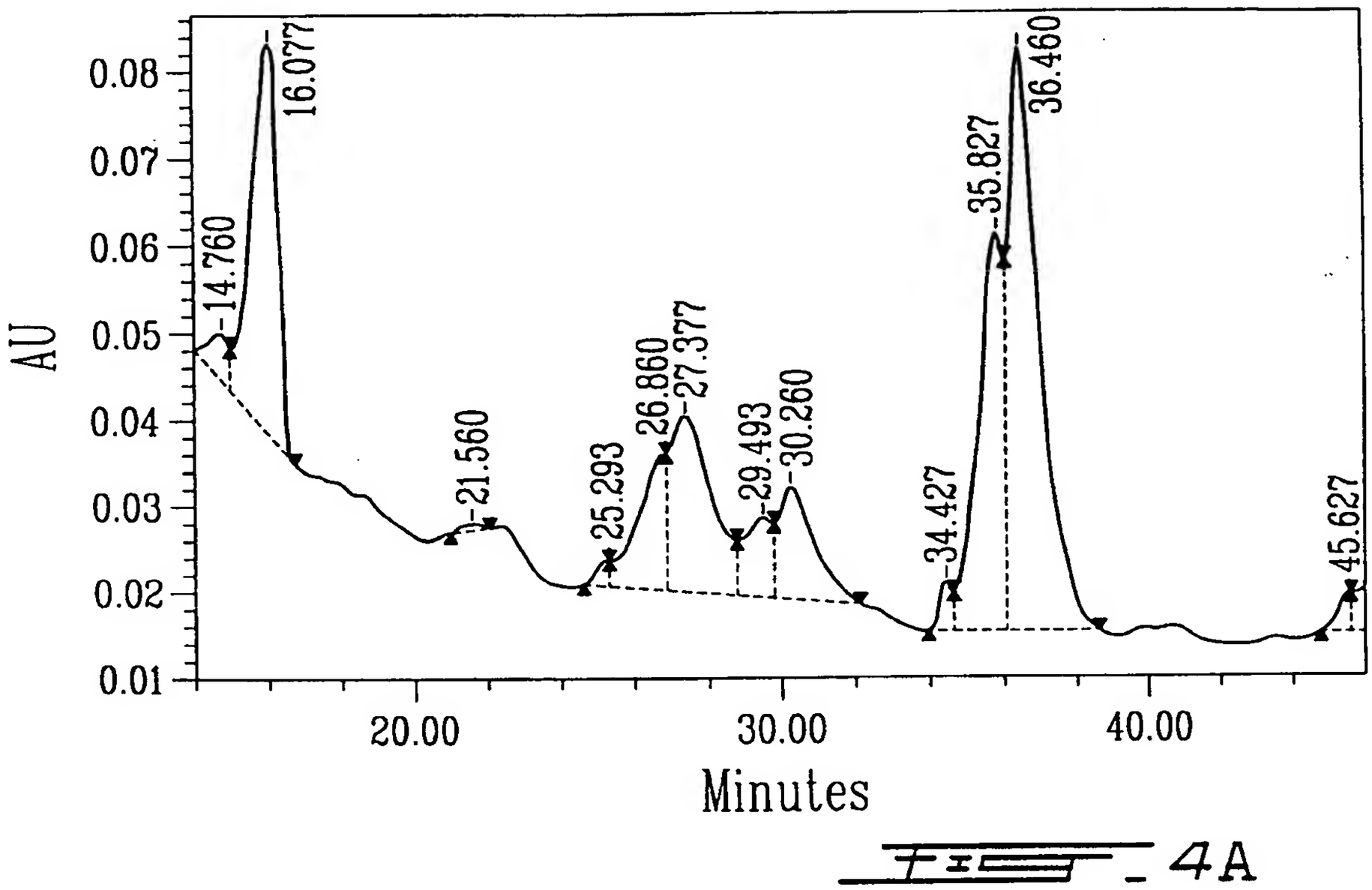

AU
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
14.760
16.077
21.560
25.293
26.860
27.377
29.493
30.260
34.427
35.827
36.460
45.627
20.00
30.00
40.00
Minutes

FIG 4A

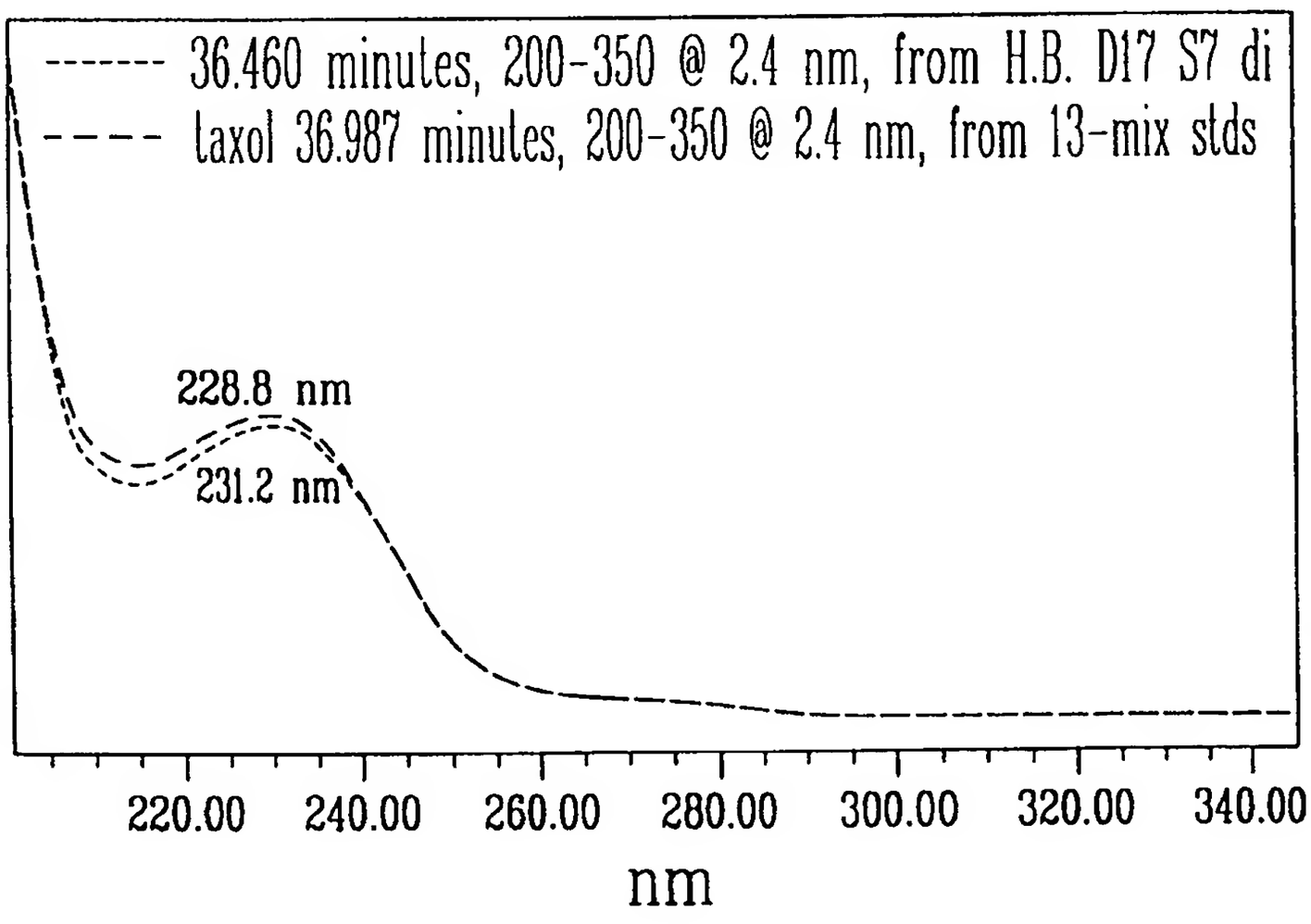

------ 36.460 minutes, 200-350 @ 2.4 nm, from H.B. D17 S7 di
- - - - taxol 36.987 minutes, 200-350 @ 2.4 nm, from 13-mix stds
228.8 nm
231.2 nm
220.00
240.00
260.00
280.00
300.00
320.00
340.00
nm

FIG 4B

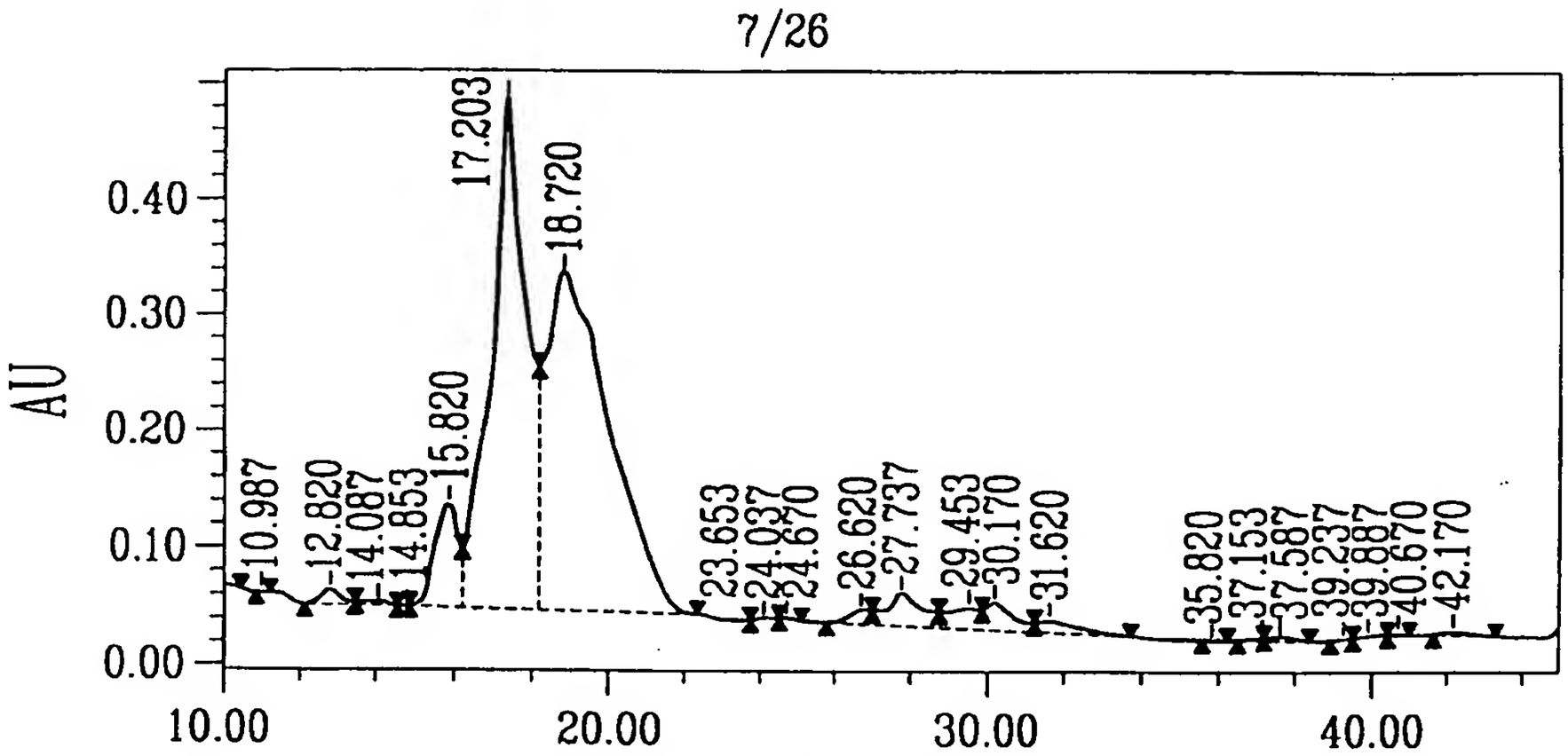
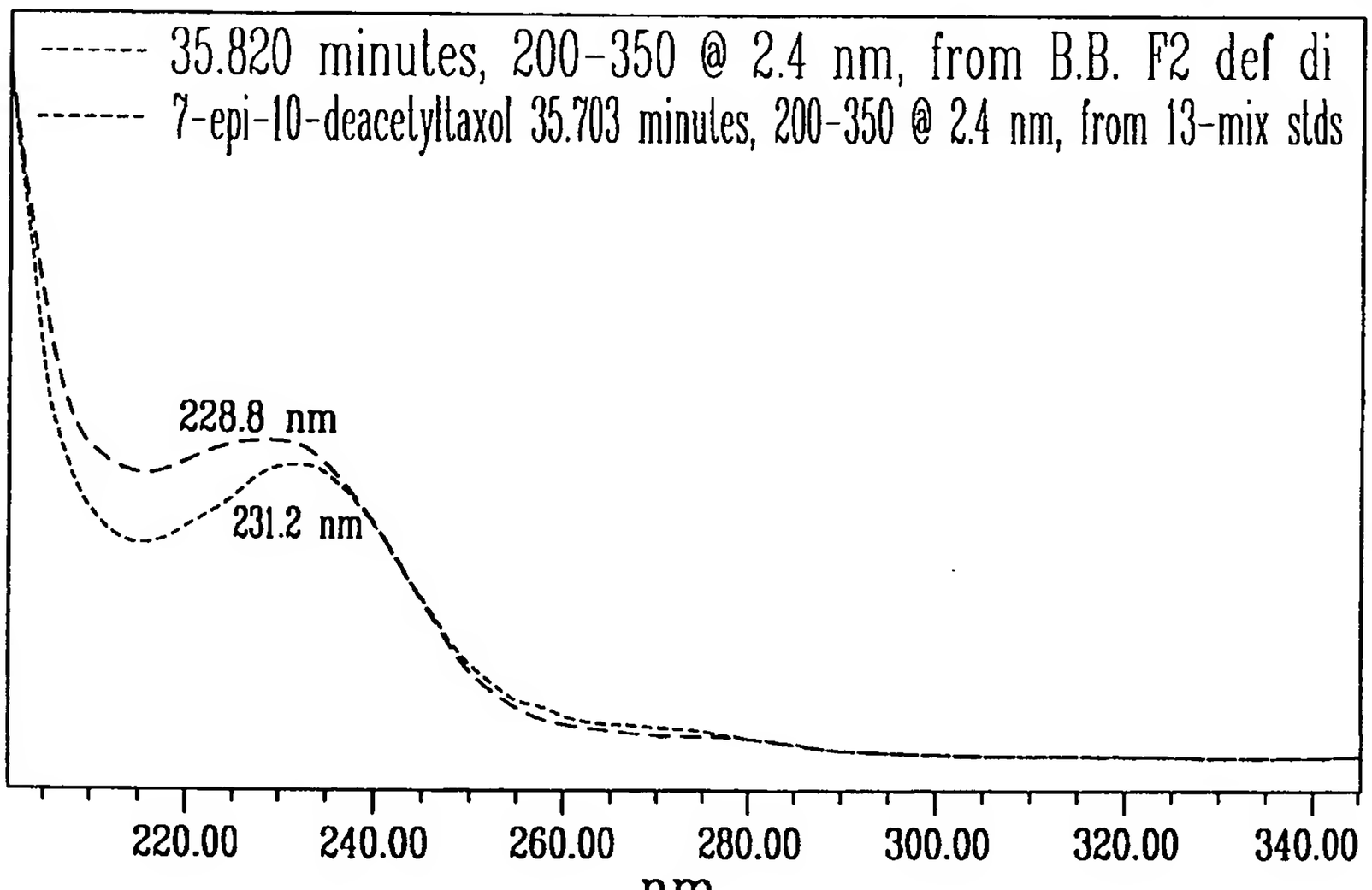


FIG - 5A



Spectral Table

#	Retention Time	Source	Spectrum Name	Baseline Correct	Searchable	Traceable
1	35.820	B.B. F2 def di		On	Yes	Yes
2	35.703	13-mix stds	7-epi-10-deacetyltaxol	On	Yes	Yes

Spectral Table

#	Start Wvln	End Wvln	Resolution	Smooth	Derivative	Spline	Lambda Max	Maximum Absorbance
1	200	350	2.4	None	None	Off	200.6	0.00501
2	200	350	2.4	None	None	Off	200.6	0.42673

FIG - 5B

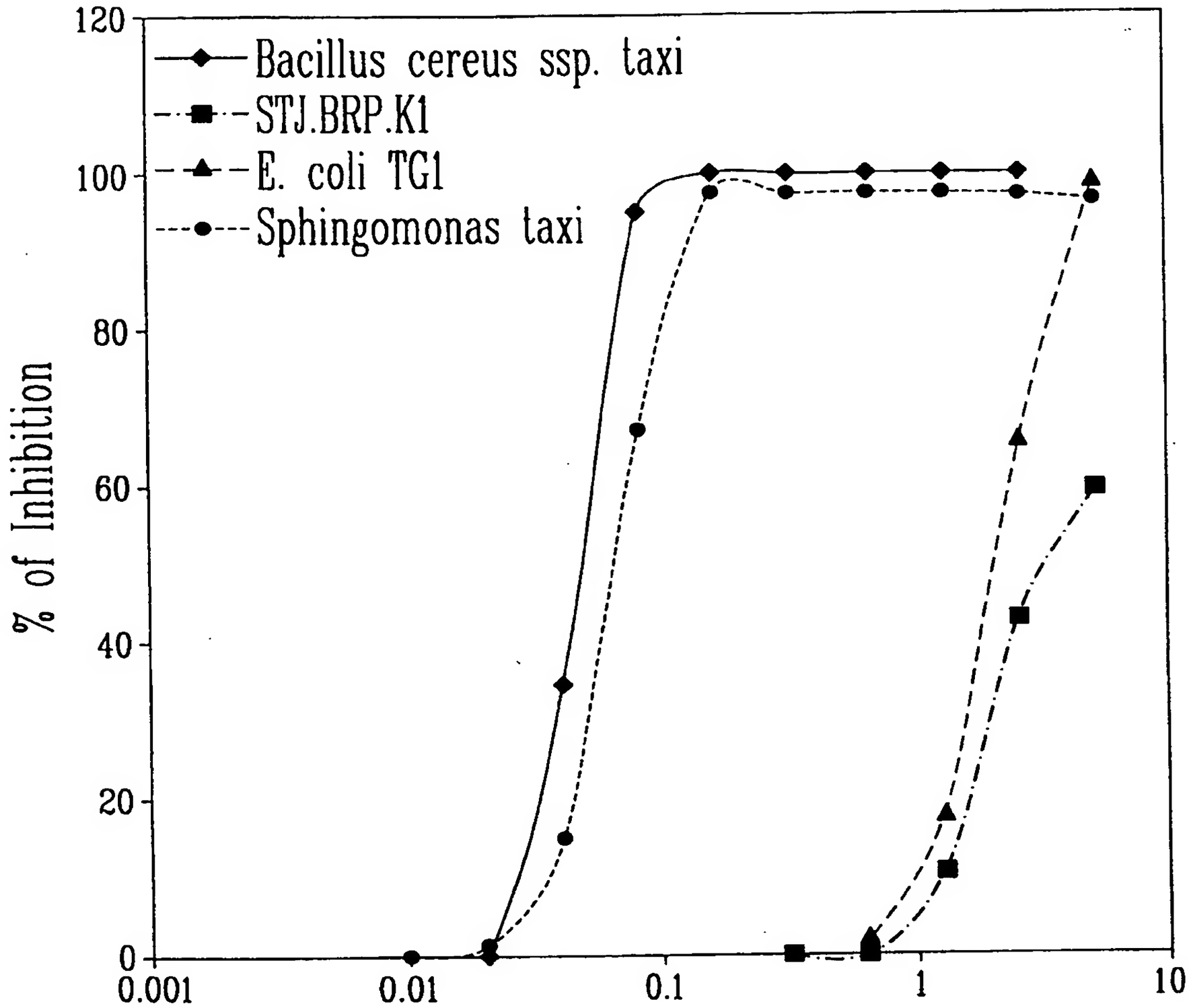

120
100
80
60
40
20
0
Bacillus cereus ssp. taxi
STJ.BRP.K1
E. coli TG1
Sphingomonas taxi
% of Inhibition
0.001
0.01
0.1
1
10
% v/v of microbial extract

Fig 6

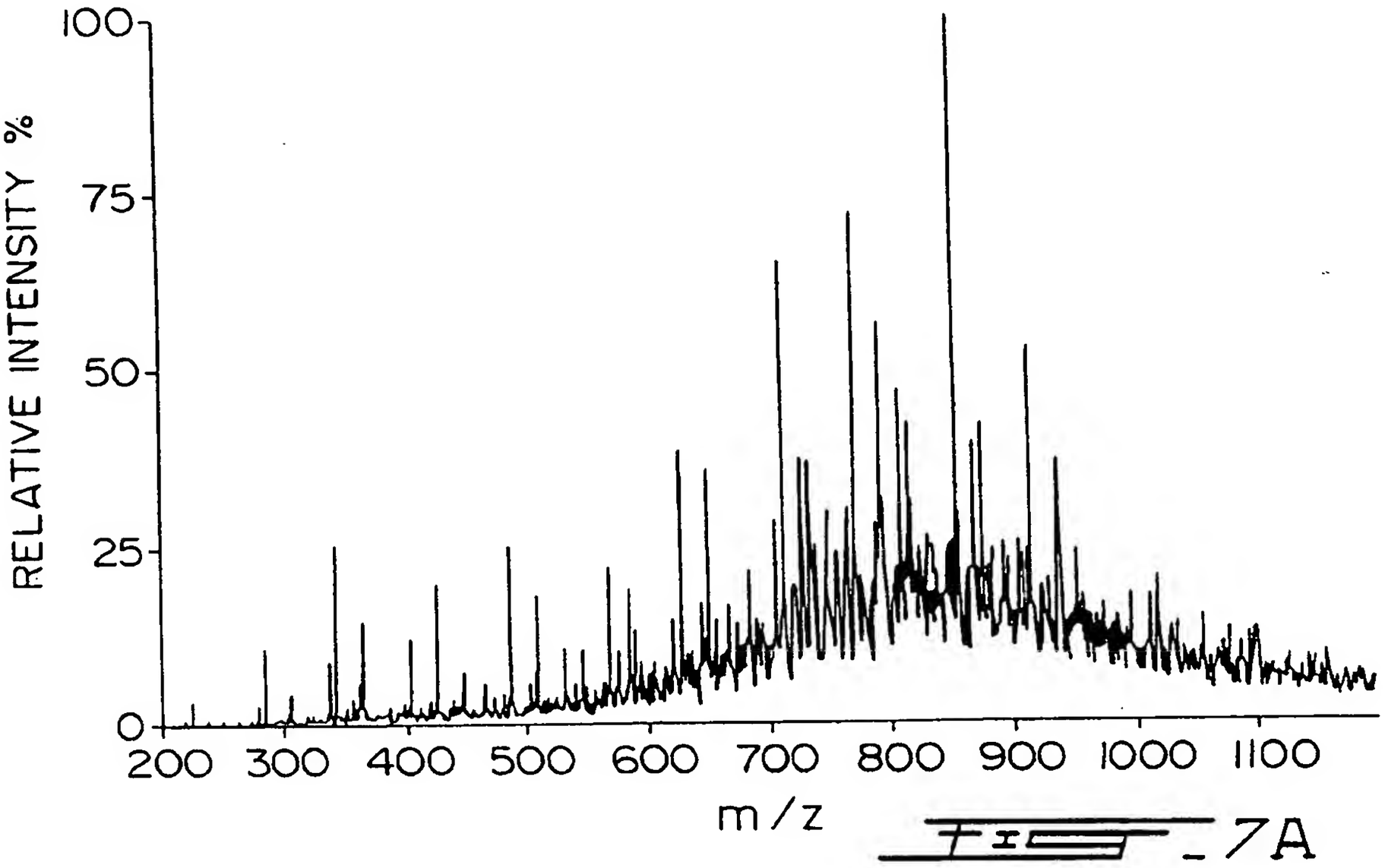

RELATIVE INTENSITY %
100
75
50
25
0
200 300 400 500 600 700 800 900 1000 1100
m/z

Fig. 7A

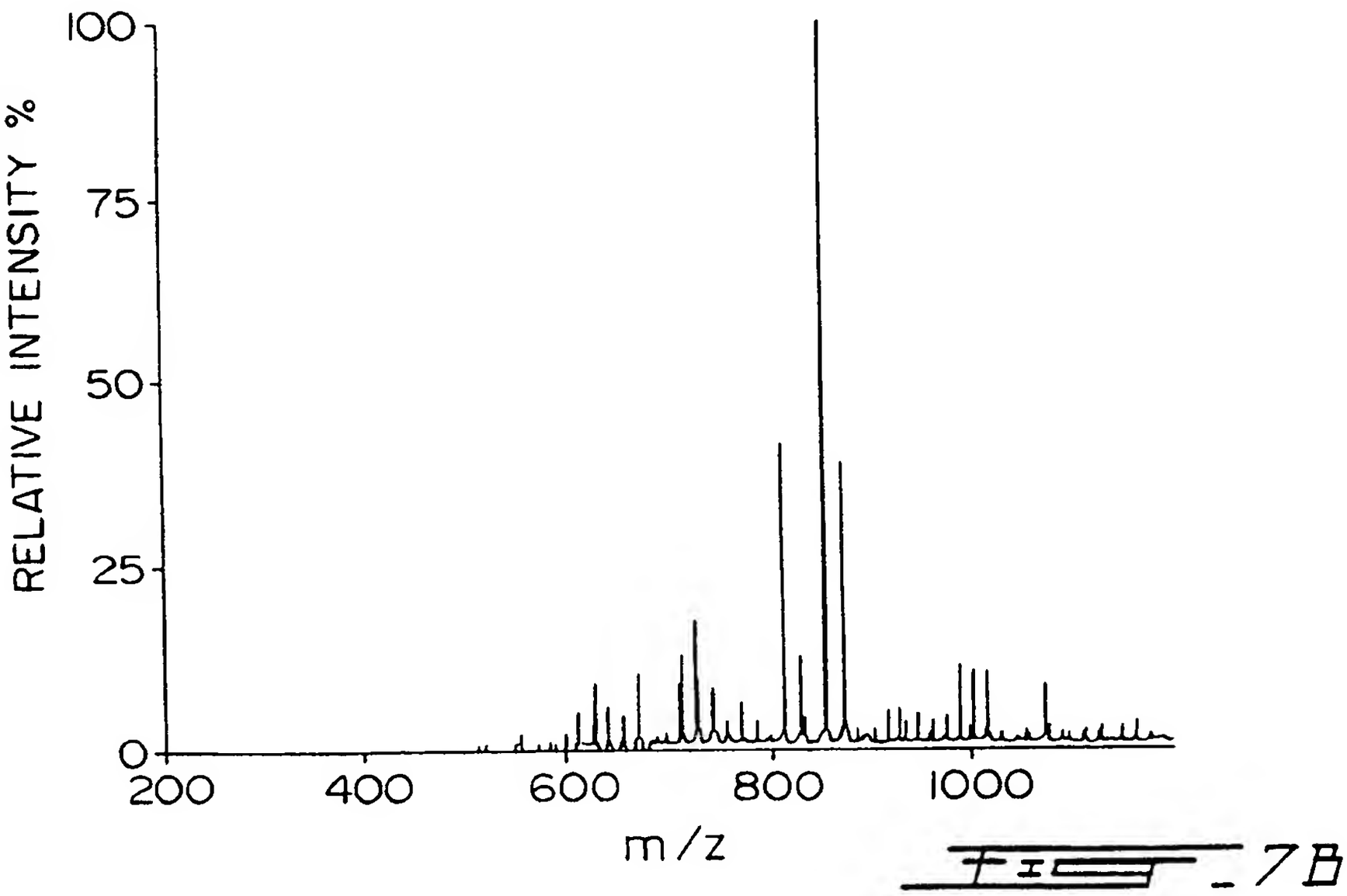

RELATIVE INTENSITY %
100
75
50
25
0
200 400 600 800 1000
m/z

Fig. 7B

```
ATGATTACGC CAAGCTATTT AGGTGACACT ATAGAATACT CAAGCTATGC ATCCAACGCG    60
TTGGGAGCTC TCCCATATGG TCGACCTGCA GGCGGCCGCA CTAGTGATTA GAGTTTGATC   120
CTGGCTCAGA ACGAACGCTG GCGGCATGCC TAACACATGC AAGTCGAACG AGATCTTCGG   180
ATCTAGTGGC GCACGGGTGC GTAACGCGTG GGAATCTGCC CTTTGGTTCG GAATAACAGT   240
TGGAAACGAC TGCTAATACC GGATGATGAC GTAAGTCCAA AGATTTATCG CCAGAGGATG   300
AGCCCGCGTA GGATTAGCTA GTTGGTGTGG TAAGAGCGCA CCAAGGCGAC GATCCTTAGC   360
TGGTCTGAGA GGATGATCAG CCACACTGGG ACTGAGACAC GGCCCAGACT CCTACGGGAG   420
GCAGCAGTGG GGAATATTGG ACAATGGGCG AAAGCCTGAT CCAGCAATGC CGCGTGAGTT   480
GATGAAAGCC TTAGGTTGTT AAAGCTCTTT TACCCGGGAA TGATAATGAC AGTACCGGGA   540
GAATAAGCTC CGGCTAACTC CGTGCCAGCA GCCGCGGTAA TACGGAAGGA GCTAGCGTTG   600
TTCGGAATTA CTGGGCGTAA AGCGCACGTA GGCGGCTTTG TAAGTTAGAG GTGAAAGCCT   660
GGAGCTCAAC TCCAGAATTG CCTTTAAGAC TGCATCGCTT GAATCCAGGA GAGGTGAGTG   720
GAATTCCGAG TGTAGAGGTG AAATTCGTAG ATATTCGGAA GAACACCAGT GGCGAAGGCG   780
GCTCACTGGA CTGGTATTGA CGCTGAGGTG CGAAAGCGTG GGGAGCAAAC AGGATTAGAT   840
ACCCTGGTAG TCCACGCCGT AAACGATGAT AACTAGCTGT CCGGGGACTT GGTCTTTGGG   900
TGGCGCAGCT AACGCATTAA GTTATCCGCC TGGGGAGTAC GGCCGCAAGG TTAAAACTCA   960
AATGAATTGA CGGGGGCCTG CACAAGCGGT GGAGCATGTG GTTTAATTCG AAGCAACGCG  1020
CAGAACCTTA CCAGCGTTTG ACATGTCCGG ACGATTTCTG GAGACAGATC TCTTCCCTTC  1080
GGGGACTGGA ACGCAGGTGC TGCATGGCTG TCGTCAGCTC GTGTCGTGAG ATGTTGGGTT  1140
AAGTCCCGCA ACGAGCGCAA CCCTCGCCTT TAGTTACCAT CATTTAGTTG GGGACTCTAA  1200
AGGAACCGCC GGTGATAAGC CGGAGGAAGG TGGGGATGAC GTCAAGTCCT CATGGCCCTT  1260
ACGCGCTGGG CTACACACGT GCTACAATGG CGGTGACAGT GGGCAGCAAA CTCGCGAGAG  1320
TGCGCTAATC TCCAAAAGCC GTCTCAGTTC GGATTGTTCT CTGCAACTCG AGAGCATGAA  1380
GGCGGAATCG CTAGTAATCG CGGATCAGCA TGCCGCGGTG AATACGTTCC CAGGCCTTGT  1440
ACACACCGCC CGTCACACCA TGGGAGTTGG GTTCACCCGA AGGCGTTGCG CTAACTCGTA  1500
AGAGAGGCAG GCGACCACGG TGGGCTTAGC GACTGGGGTG AAGTCGTAAC AAGGTA      1556
```

FIG. 8A

```
AGAGTTTGAT CATGGCTCAG GATGAACGCT GGCGGCGTGC CTAATACATG CAAGTCGAGC    60
GAATGGATTA AGAGCTTGCT CTTATGAAGT TAGCGGCGGA CGGGTGAGTA ACACGTGGGT   120
AACCTACCCA TAAGACTGGG ATAACTCCGG GAAACCGGGG CTAATACCGG ATAATATTTT   180
GAACTGCATA GTTCGAAATT GAAAGGCGGC TTCGGCTGTC ACTTATGGAT GGACCCGCGT   240
CGCATTAGCT AGTTGGTGAG GTAACGGCTC ACCAAGGCGA CGATGCGTAG CCGACCTGAG   300
AGGGTGATCG GCCACACTGG GACTGAGACA CGGCCCAGAC TCCTACGGGA GGCAGCAGTA   360
GGGAATCTTC CGCAATGGAC GAAAGTCTGA CGGAGCAACG CCGCGTGAGT GATGAAGGCT   420
TTCGGGTCGT AAAACTCTGT TGTTAGGGAA GAACAAGTGC TAGTTGAATA AGCTGGCACC   480
TTGACGGTAC CTAACCAGAA AGCCACGGCT AACTACGTGC CAGCAGCCCG CGGTAATACG   540
TAGGTGGCAA GCGTTATCCG GAATTATTGG GCGTAAAGCG CGCGCAGGTG GTTTCTTAAG   600
TCTGATGTGA AAGCCCACGG CTCAACCGTG GAGGGTCATT GGAAACTGGG AgACTTGAGT   660
GCAGAAGAGG AAAGTGGAAT TCCATGTGTA GCGGTGAAAT GCGTAgAGAT ATGGAGGAAC   720
ACCAGTGGCG AAGGCGACTT TCTGGTCTGT AACTGACACT GAGGCGCGAA AGCGTGGGGA   780
GCAAACAGGA TTAGATACCC TGGTAGTCCA CGCCGTAAAC GATGAGTGCT AAGTGTTAGA   840
GGGTTTCCGC CCTTTAGTGC TGAAGTTAAC GCATTAAGCA CTCCGCCTGG GGAGTACGGC   900
CGCAAGGCTG AAACTCAAAG GAATTGACGG GGGCCCGCAC AAGCGGTGGA GCATGTGGTT   960
TAATTCGAAG CAACGCGAAG AACCTTACCA GGTCTTGACA TCCTCTGAAA ACTCTAGAGA  1020
TAgAGCTTCT CCTTCGGGAG CAGAGTGACA GGTGGTGCAT GGTTGTCgTC AGCTCGTGTC  1080
GTGAGATGTT GGGTTAAGTC CCGCAACGAG CGCAACCCTT GATCTTAGTT GCCATCATTA  1140
AGTTGGGCAC TCTAAGGTGA CTGCCGGTGA CAAACCGGAG GAAGGTGGGG ATGACGTCAA  1200
ATCATCATGC CCCTTATGAC CTGGGCTACA CACGTGCCAC AATGGACGGT ACAAAGAGCT  1260
GCAAGACCGC GAGGTGGAGC TAATCTCATA AAACCGTTCT CAGTTCGGAT TGTAGGCTGC  1320
AACTCGCCTA CATGAAGCTG GAATCGCTAG TAATCGCGGA TCAGCATGCC GCGGTGAATA  1380
CGTTCCCGGG CCTTGTACAC ACCGCCCGTC ACACCACGAG AGTTTGTAAC ACCCGAAGTC  1440
GGTGGGGTAA CCTTTATGGA GCCAGCCGCC TAAGGTGGGA CAGATGATTG GGGTGAAGTC  1500
GTAACAAGGT AA                                                      1512
```

Fig. 8B

```
AGAGTTTGAT CCTGGCTCAG GATGAACGCT GGCGGCGTGC CTAATACATG CAAGTCGAGC    60
GAACTGATTA GAAGCTTGCT TCTATGACGT TAGCGGCGGA CGGGTGAGTA ACACGTGGGC   120
AACCTGCCTG TAAGACTGGG ATAACTTCGG GAAACCGAAG CTAATACCGG ATAGGATCTT   180
CTCCTTCATG GGGGATGATT GAAAGATGGT TTCGGCTATC ACTTACAGAT GGGCCCGCGG   240
TGCATTAGCT AGTTGGTGAG GTAACGGCTC ACCAAGGCAA CGATGCATAG CCGACCTGAG   300
AGGGTGATCG GCCACACTGG GACTGAGACA CGGCCCAAAC TCCTACGGGA GGCAGCAGTA   360
GGGAATCTTC CGCAATGGAC GAAAGTCTGA CGGAGCAACG CCGCGTGAGT GATGAAGGCT   420
TTCGGGTCCT AAAACTCTGT TGTTnGGGAA GAACAAATAC CAGAGTAACT GCTTGTTCCT   480
TGACGGTACC TAACCAGAAA GCCACGGCTA ACTACTTTCC AGCAGCCGCG GTAATACnTA   540
TGTTGGCAAG CGTTATCCGG AATTATTGGG GCGTTAAAGC GCGCGCCAGG CGGTTTCTTA   600
AGTCTGATGT GAAAGCCCAC GGCTCAACCG TGGAGGGTCA TTGGAAACTG GGGAACTTGA   660
GTGCAGAAGA GAAAAGCGGA ATTCCACGTG TAGCGGTGAA ATGCGTAGAG ATGTGGAGGA   720
ACACCAGTGG CGAAGGCGGC TTTTTGGTCT GTAACTGACG CTGAGGCGCG AAAGCGTGGG   780
GAGCAAACAG GATTAGATAC CCTGGTAGTC CACGCCGTAA ACGATGAGTG CTAAGTGTTA   840
GAGGGTTTCC GCCCTTTAGT GCTGCAGCTA ACGCATTAAG CACTCCGCCT GGGGAGTACG   900
GTCGCAAGAC TGAAACTCAA AGGAATTGAC GGGGGCCCGC ACAAGCGGTG GAGCATGTGG   960
TTTAATTCGA AGCAACGCGA ArAACCTTAC CAGGTCTTGA CATCCTCTGA CAACTCTAGA  1020
GATAGAGCGT TCCCCTTCGG GGGACAGAGT GACAGGTGGT GCATGGTTGT CGTCAGCTCG  1080
TGTCgTGAGA TgTTGGGTtA AGTCCCGCAA CGAGCGCAAC CCTTGATCTT AGTTGCCAgC  1140
ATTTAGTTGG GCACTCTAAG GTGACTGCCG GTGACAAACC GGAGGAAGGT GGGGATGACG  1200
TCAAATCATC ATGCCCCTTA TGACCTGGGC TACACACGTG CTACAATGGA TGGTACAAAG  1260
GGCTGCAAGA CCGCGAGGTC AAGCCAATCC CATAAAACCA TTCTCAGTTC GGATTGTAGG  1320
CTGCAACTCG CCTACATGAA GCTGGAATCG CTAGTAATCG CGGATCAGCA TGCCGCGGTG  1380
AATACGTTCC CGGGCCTTGT ACACACCGCC CGTCACACCA CGAGAGTTTG TAACACCCGA  1440
AGTCGGTGGA GTAACCGTAA GGAGCTAGCC GCCTAAGGTG GGACAGATGA TTGGGGTGAA  1500
GTCGTAACAA GGTAA                                                   1515
```

Fig. 8C

```
TCGGGACGGT CAGCACACGA GGAGCTTGCT CCTTGGGTGA CGAGTGGCGG ACGGGTGAGT    60
AATGTCTGGG GATCTGCCCG ATAnAGGGGG ATAACCACTG GAAACGGTGG CTAATACCGC   120
ATAACGTCGC AAGACCAAAG AGGGGGACCT TCGGGCCTCT CACTATCGGA TGAACCCAGA   180
TGGGATTAGC TAGTAnGCGG GGTAATGGCC CACCTAGGCG ACGATCCCTA nCTGGTCTGA   240
GAGGATGACC AGCCACACTG GAACTGAGAC ACGGTCCAnA CTCCTACGGG AGGCAGCAGT   300
GGGGAATATT GCACAATGGG CGCAAGCCTG ATGCAGCCAT GCCGCGTGTA TGAAGAAGGC   360
CTTCGGGTTG TAAAGTACTT TCAGCGGGGA GGAAGGCGAT GCGGTTAATA ACCCTGTCGA   420
TTGACGTTCC CCGCAnGAAG AAGCACCGGC TAACTCCGTG CCAGCAGCCG CGGTAATACC   480
GGAGGGTGCA AGCGTTAATC CGGAATTACT GGGCGTAAAG CGCACGCAGG CGGTCTGTTA   540
AGTCAGATGT GAAATCCCCG GGCTTAACCT GGGAACTGCA TTTGAAACTG GCAGGCTTGA   600
GTCTTGTAGA GGGGGGTAGA ATTCCAGGTG TAGCGGTGAA ATGCGTAGAG ATCTGGAGGA   660
ATACCGGTGG CGAAGGCGGC CCCTTGGACA AAGACTGACG CTCAGGTGCG AAAGCGTGGG   720
GAGCAAACAG GATTAGATAC CCTGGTAGTC CACGCCGTAA ACGATGTCGA CTTGGAGGTT   780
GTTCCCTTGA GGAGTGGCTT CCGGAGCTAA CGCGTTAAGT CGACCGCCTG GGGAGTACGG   840
CCGCAAGGTT AAAATTCAAA TGAATTGACG GGGGCCCGCA CAAGCGGTGG AGCATGTGGT   900
TTAATTCGAT GCAACGCGAA GAACCTTACC TACTCTTGAC ATCCAGCGAA CTTGCCAGAG   960
ATGGATTGGT GCCTTCGGGA ACGCTGAGAC AGGTGCTGCA TGGCTGTCGT CAGCTCGTGT  1020
TGTGAAATGT TGGGTTAAGT CCCGCAACGA GCGCAACCCT TATCCTTTGT TGCCAGCGAT  1080
TCGGTCGGGA ACTCAAAGGA GACTGCCGGT GATAAACCGG AGGAAGGTGG GGATGACGTC  1140
AAGTCATCAT GGCCCTTACG AGTAGGGCTA CACACGTGCT ACAATGGCGC ATACAAAGAG  1200
AAGCGACCTC GCGAGAGCAA GCGGACCTCA CAAAGTGCGT CGTAGTCCGG ATCGGAGTCT  1260
GCAACTCGAC TCCGTGAAGT CGGAATCGCT AGTAATCGTG GATCAGAATG CCACGGTGAA  1320
TACGTTCCCG GGCCTTGTAC ACACCGCCCG TCACACCATG GGAGTGGGTT GCAAAAGAAG  1380
TAGGTAGCTT AACCTTCGGG AGGGCGCTTA CCACTTTGTG ATTCATGACT GGGGTGAAGT  1440
CGTAACAAGT A                                                       1451
```

Fig. 8D

```
AGAGTTTGAT CCTGGCTCAG GATGAACGCT GGCGGCGTGC CTAATACATG CAAGTCGAGC    60
GAATGGATTG AGAGCTTGCT CTCAAGAAGT TAGCGGCGGA CGGGTGAGTA ACACGTGGGT   120
AACCTGCCCA TAAGACTGGG ATAACTCCGG GAAACCGGGG CTAATACCGG ATAACATTTT   180
GAACTGCATG GTTCGAAATT GAAAGGCGGC TTCGGCTGTC ACTTATGGAT GGACCCGCGT   240
CGCATTAGCT AGTTGGTGAG GTAACGGCTC ACCAAGGCAA CGATGCGTAG CCGACCTGAG   300
AGGGTGATCG GCCACACTGG GACTGAGACA CGGCCCAGAC TCCTACGGGA GGCAGCAGTA   360
GGGAATCTTC CGCAATGGAC GAAAGTCTGA CGGAGCAACG CCGCGTGAGT GATGAAGGCT   420
TTCGGGTCGT AAAACTCTGT TGTTAGGGAA GAACAAGTGC TAGTTGAATA AGCTGGCACC   480
TTGACGGTAC CTAACCAGAA AGCCACGGCT AACTACGTGC CAGCAGCCGC GGTAATACGT   540
AGGTGGCAAG CGTTATCCGG AATTATTGGG CGTAAAGCGC GCGCAGGTGG TTTCTTAGGT   600
CTGATGTGAA AGCCCACGGC TCAACCGTGG AGGGTCATTG GAAACTGGGA GACTTGAGTG   660
CAGAAGAGGA AAGTGGAATT CCATGTGTAG CGGTGAAATG CGTAGAGATA TGGAGGAACA   720
CCAGTGGCGA AGGCGACTTT CTGGTCTGTA ACTGACACTG AGGCGCGAAA GCGTGGGGAG   780
CAAACAGGAT TAGATACCCT GGTAGTCCAC GCCGTAAACG ATGAGTGCTA AGTGTTAGAG   840
GGTTTCCGCC CTTTAGTGCT GAAGTTAACG CATTAAGCAC TCCGCCTGGG GAGTACGGCC   900
GCAAGGCTGA AACTCAAAGG AATTGACGGG GGCCCGCACA AGCGGTGGAG CATGTGGTTT   960
AATTCGAAGC AACGCGAAGA ACCTTACCAG GTCTTGACAT CCTCTGAAAA CCCTAGAGAT  1020
AGGGCTTCTC CTTCGGGAGC AGAGTGACAG GTGGTGCATG GTTGTCGTCA GCTCGTGTCG  1080
TGAGATGTTG GGTTAAGTCC CGCAACGAGC GCAACCCTTG ATCTTAGTTG CCATCATTAA  1140
GTTGGGCACT CTAAGGTGAC TGCCGGTGAC AAACCGGAGG AAGGTGGGGA TGACGTCAAA  1200
TCATCATGyC CCTTATGACC TGGGCTACAC ACGTGCTACA ATGGACGGTA CAAAGAGCTG  1260
CAAGACCGCG AGGTGGAGCT AATCTCATAA AACCGTTCTC AGTTCrGATT GTAGGCTGCA  1320
ACTCGCCTAC ATGAAGCTGG AATCGCTAGT AATCGCGGAT CAGCATGCCG CGGTGAATAC  1380
GTTCCCGGGC CTTGTACACA CCGCCCGTCA CACCACGAGA GTTTGTAACA CCCGAAGTCG  1440
GTGGGGTAAC CTTTTTGGAG CCAGCCGCCT AAGGTGGGAC AGATGATTGG GGTGAAGTCG  1500
TAACAAGGTA GCC                                                     1513
```


FIG. 8E

```
AGAGTTTGAT CCTGGCTCAG GACGAACGCT GGCGGCGTGC CTAATACATG CAAGTCGAGC    60
GAACAGATGG GAGCTTGCTC CCTGATGTTA GCGGCGGACG GGTGAGTAAC ACGTGGGTAA   120
CCTGCCTGTA AGACTGGGAT AACTCCGGGA AATCGGGGCT AATACCGGAT GGTTGTTTGA   180
ACCGCATGGT TCAGACATAA AAGGTGGCTT CGGCTACCAC TTACAGATGG ACCCGCGGCG   240
CATTAGCTAG TTGGTGAGGT AACGGCTCAC CAAGGCGACG ATGCGTAGCC GACCTGAGAG   300
GGTGATCGGC CACACTGGGA CTGAGACACG GCCCAGACTC CTACGGGAGG CAGCAGTAGG   360
GAATCTTCCG CAATGGACGA AAGTCTGACG GAGCAACGCC GCGTGAGTGA TGAAGGTTTT   420
CGGATCGTAA AGCTCTGTTG TTAGGGAAGA ACAAGTGCCG TTCAAATAGG GCGGCACCTT   480
GACGGTACCT AACCAGAAAG CCACGGCTAA CTACGTGCCA GCAGCCGCGG TAATACGTAG   540
GTGGCAAGCG TTGTCCGGAA TTATTGGGCG TAAAGGGCTC GCAGGCGGTT TCTTAAGTCT   600
GATGTGAAAG CCCCCGGTTC AACGGGGGAG GGTCATTGGA AACTGGGGAA CTTGAGTGCA   660
GAAGAGGAGA GTGGAATTCC ACGTGTAGCG GTGAAATGCG TAGAGATGTG GAGGAACACC   720
AGTGGCGAAG GCGACTCTCT GGTCTGTAAC TGACGCTGAG GAGCGAAAGC GTGGGGAGCG   780
AACAGGATTA GATACCCTGG TAGTCCACGC CGTAAACGAT GAGTGCTAAG TGTTAGGGGG   840
TTTCCGCCCC TTAGTGCTGC AGCTAACGCA TTAAGCACTC CGCCTGGGGA GTACGGTCGC   900
AAGACTGAAA CTCAAAGGAA TTGACGGGGG CCCGCACAAG CGGTGGAGCA TGTGGTTTAA   960
TTCGAAGCAA CGCGAAGAAC CTTACCAGGT CTTGACATCC TCTGACAATC CTAGAGATAG  1020
GACGTCCCCT TCGGGGGCAG AGTGACAGGT GGTGCATGGT TGTCGTCAGC TCGTGTCGTG  1080
AGATGTTGGG TTAAGTCCCG CAACGAGCGC AACCCTTGAT CTTAGTTGCC AGCATTCAGT  1140
TGGGCACTCT AAGGTGACTG CCGGTGACAA ACCGGAGGAA GGTGGGGATG ACGTCAAATC  1200
ATCATGCCCC TTATGACCTG GGCTACACAC GTGCTACAAT GGGCAGAACA AAGGGCAGCG  1260
AAACCGCGAG GTTAAGCCAA TCCCACAAAT CTGTTCTCAG TTCGGATCGC AGTCTGCAAC  1320
TCGACTGCGT GAAGCTGGAA TCGCTAGTAA TCGCGGATCA GCATGCCGCG GTGAATACGT  1380
TCCCGGGCCT TGTACACACC GCCCGTCACA CCACGAGAGT TTGTAACACC CGAAGTCGGT  1440
GAGGTAACCT TTATGGAGCC AGCCGCCGAA GGTGGGACAG ATGATTGGGG TGAAGTCGTA  1500
ACAAGGTAAC CG                                                       1512
```

FIG. 8F

```
GCCTAACAGT GCGAGTCGGA CGGTAGCACA GAGGAGCTTG CTCTTCGGGT GACGAGTGGC    60
GGACGGGTGA GTAATGTCTG GGGATCTGCC CGATGGAGGG GGATAACCAC TGGAAACGGT   120
GGCTAATACC GCATAATGTC GCAAGACCAA AGTGGGGGAC CTTCGGGCCT CACACCATCG   180
GATGAACCCA GATGGGATTA GCTAGTAGGT GGGGTAACGG CTCACCTAGG CGACGATCCC   240
TAGCTGGTCT GAGAGGATGA CCAGCCACAC TGGAACTGAG ACACGGTCCA GACTCCTACG   300
GGAGGCAGCA GTGGGGAATA TTGCACAATG GGCGCAAGCC TGATGCAGCC ATGCCGCGTG   360
TATGAAGAAG GCCTTCGGGT TGTAAAGTAC TTTCAnCGGG GAGGAAGGGG ACGAGGTTAA   420
TAACCCCGTT CATTGACGTT ACCCGCAGAA GAAGCACCGG CTAACTCCGT GCCAGCAGCC   480
GCGGTAATAC GGAGGGTGCA AGCGTTAATC GGAATTACTG GGCGTAAAGC GCACGCAGGC   540
GGTCTGTTAA GTCAGATGTG AAATCCCCGG GCTTAACCTG GGAACTGCAT TTGAAACTGG   600
CAGGCTTGAG TCTTGTAGAG GGGGGTAGAA TTCCAGGTGT AGCGGTGAAA TGCGTAGAGA   660
TCTGGAGGAA TACCGGTGGC GAAGGCGGCC CCCTGGACAA AGACTGACGT TCAGGTGCGA   720
AAGCGTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCCGTAAA CGATGTCGAC   780
TTGGAGGCTG TGAGCATGAT TCGTGGCTTC CGGAGCTAAC GCGTTAAGTC GACCGCCTGG   840
GGAGTACGGC CGCAAGGTTA AAATTCAAAT GAATTGACGG GGGCCCGCAC AAGCGGTGGA   900
GCATGTGGTT TAATTcGATG CAACGCGAAG AACCTTACCT GyTCTTGACA TCCACGGAAT   960
TyGGCAGAGA TGCCTTAGTG CCTTCGGGAA CCGTGAGACA GGTGCTGCAT GGCTGTCGTC  1020
AGCTCGTGTT GTGAAATGTT GGGTTAAGTC CCGCAACGAG CGCAACCCTT ATCCTTTGTT  1080
GCCAGCGATT CGGTCGGGAA CTCAAAGGAG ACTGCCGGTG ATAAACCGGA GGAAGGTGGG  1140
GATGACGTCA AGTCATCATG GCCCTTACGA GCAGGGCTAC ACACGTGCTA CAATGGCGCA  1200
TACAAAGAGA AGCGACCTCG CGAGAGCAAG CGGACCTCAC AAAGTGCGTC GTAGTCCGGA  1260
TCGGAGTCTG CAACTCGACT CCGTGAAGTC GGAATCGCTA GTAATCGTGG ATCAGAATGC  1320
CACGGTGAAT ACGTTCCCGG GCCTTGTACA CACCGCCCGT CACACCATGG GAGTGGGTTG  1380
CAAAAGAAGT AGGTAGCTTA ACCTTCGGGA GGGCGCTTAC CACTTTGTGA TTCATGACTG  1440
GGGTGAAGTC GTAACAAG                                                1458
```

Fig. 8G

```
AGTGCGAGTC GGACGGTAGC ACAGAGAGCT TGCTCTTGGG TGACGAGTGG CGGACGGGTG    60
AGTAATGTCT GGGGATCTGC CCGATAGAGG GGGATAACCA CTGGAAACGG TGGCTAATAC   120
CGCATAACGT CGCAAGACCA AAGAGGGGGA CCTTCGGGCC TCTCACTATC GGATGAACCC   180
AGATGGGATT AGCTAGTAGG CGGGGTAATG GCCCACCTAG GCGACGATCC CTAGCTGGTC   240
TGAGAGGATG ACCAGCCACA CTGGAACTGA GACACGGTCC AGACTCCTAC GGGAGGCAGC   300
AGTGGGGAAT ATTGCACAAT GGGCGCAAGC CTGATGCAGC CATGCCGCGT GTATGAAGAA   360
GGCCTTCGGG TTGTAAAGTA CTTTCAGCGG GGAGGAAGGC GATGCGGTTA ATAACCCTGT   420
CGATTGACGT TACCCGCAGA AGAAGCACCG GCTAACTCCG TGCCAGCAGC CGCGGTAATA   480
CGGAGGGTGC AAGCGTTAAT CGGAATTACT GGGCGTAAAG CGCACGCAGG CGGTCTGTTA   540
AGTCAGATGT GAAATCCCCG GGCTTAACCT GGGAACTGCA TTTGAAACTG GCAGGCTTGA   600
GTCTTGTAGA rGGGGGTAGA ATTCCAGGTG TAGCGGTGAA ATGCGTAGAG ATCTGGAGGA   660
ATACCGGTGG CGAAGGCGGC CCCCTGGACA AAGACTGACG CTCAGGTGCG AAAGCGTGGG   720
GAGCAAACAG GATTAGATAC CCTGGTAGTC CACGCCGTAA ACGATGTCGA CTTGGAGGTT   780
GTTCCCTTGA GGAGTGGCTT CCGGAGCTAA CGCGTTAAGT CGACCGCCTG GGGAGTACGG   840
CCGCAAGGTT AAAATTCAAA TGAATTGACG GGGGCCCGCA CAAGCGGTGG AGCATGTGGT   900
TTAATTCGAT GCAACGCGAA GAACCTTACC TACTCTTGAC ATCCACGGAA TTyGGCAGAG   960
ATGCTTTGGT GCCTTCGGGA ACCCTGAGAC AGGTGCTGCA TGGCTGTCGT CAGCTCGTGT  1020
TGTGAAATGT TGGGTTAAGT CCCGCAACGA GCGCAACCCT TATCCTTTGT TGCCAGCGAT  1080
TCGGTCGGGA ACTCAAAGGA GACTGCCGGT GATAAACCGG AGGAAGGTGG GGATGACGTC  1140
AAGTCATCAT GGCCCTTACG AGTAGGGCTA CACACGTGCT ACAATGGCGC ATACAAAGAG  1200
AAGCGACCTC GCGAGAGCAA GCGGACCTCA CAAAGTGCGT CGTAGTCCGG ATCGGAGTCT  1260
GCAACTCGAC TCCGTGAAGT CGGAATCGCT AGTAATCGTG GATCAGAATG CCACGGTGAA  1320
TACGTTCCCG GGCCTTGTAC ACACCGCCCG TCACACCATG GGAGTGGGTT GCAAAAGAAG  1380
TAGGTAGCTT AACCTTCGGG AGGGCGCTTA CCACTTTGTG ATTCATGACT GGGGTGAAGT  1440
CGTAACAAGT A                                                       1451
```

FIG. 8H

```
AGAGTTTGAT CATGGCTCAG GATGAACGCT GGCGGCGTGC CTAATACATG CAAGTCGAGC    60
GAACTGATTA GAAGCTTGCT TCTATGACGT TAGCGGCGGA CGGGTGAGTA ACACGTGGGC   120
AACCTGCCTG TAAGACTGGG ATAACTTCGG GAAACCGAAG CTAATACCGG ATAGGATCTT   180
CTCCTTCATG GGAGATGATT GAAAGATGGT TTCGGCTATC ACTTACAGAT GGGCCCGCGG   240
TGCATTAGCT AGTTGGTGAG GTAACGGCCC ACCAAGGCAA CGATGCATAG CCGACCTGAG   300
AGGGTGATCG GCCACACTGG GACTGAGACA CGGCCCAGAC TCCTACGGGA GGCAGCAGTA   360
GGGAATCTTC CGCAATGGAC GAAAGTCTGA CGGAGCAACG CCGCGTGAGT GATGAAGGCT   420
TTCGGGTCGT AAAACTCTGT TGTTAGGGAA GAACAAGTAC GAGAGTAACT GCTCGTACCT   480
TGACGGTACC TAACCAGAAA GCCACGGCTA ACTACGTGCC AGCAGCCGCG GTAATACGTA   540
GGTGGCAAGC GTTATCCGGA ATTATTGGGC GTAAAGCGCG CCGCAGGCGG TTTCTTAAGT   600
CTGATGTGAA AGCCCACGGC TCAACCGTgG GArGGTCATT GGAAACTGGG GAACTTGAgT   660
GCAGAAGAGA AAAGCGGAAT TCCACGTGTA GCGGTGAAAT GCGTAGAGAT GTGGAGGAAC   720
ACCAGTGGCG AAGGCGGCTT TTTGGTCTGT AACTGACGCT GAGGCGCGAA AGCGTGGGGA   780
GCAAACAGGA TTAGATACCC TGGTAGTCCA CGCCGTAAAC GATGAGTGCT AAGTGTTAGA   840
GGGTTTCCGC CCTTTAGTGC TGCAGCTAAC GCATTAAGCA CTCCGCCTGG GGAGTACGGT   900
CGCAAGACTG AAACTCAAAG GAATTGACGG GGGCCCGCAC AAGCGGTGGA GCATGTGGTT   960
TAATTCGAAG cAACGCGAAG AACCTTACCA GGTCTTGACA TCcTCTGACA ACTCTAGAGA  1020
TAGAGCtTTC CCcTTCGGGG GACAGAGTGA CAGGTGGTGC ATGGTTGTCG TCAGCTCGTG  1080
TCGTGAGATG TTGGGTTAAG TCCCGCAACG AGCGCAACCC TTGATCTTAG TTGCCAGCAT  1140
TTAGTTGGGC ACTCTAAGGT GACTGCCGGT GACAAACCGG AGGAAGGTGG GGATGACGTC  1200
AAATCATCAT GCCCCTTATG ACCTGGGCTA CACACGTGCT ACAATGGATG GTACAAAGGG  1260
CTGCAAGACC GCGAGGTCAA GCCAATCCCA TAAAACCATT CTCAGTTCGG ATTGTAGGCT  1320
GCAACTCGCC TACATGAAGC TGGAATCGCT AGTAATCGCG GATCAGCATG CCGCGGTGAA  1380
TACGTTCCCG GGCCTTGTAC ACACCGCCCG TCACACCACG AGAGTTTGTA ACACCCGAAG  1440
TCGGTGGAGT AACCGTAAGG AGCTAGCCGC CTAAGGTGGG ACAGATGATT GGGGTGAAGT  1500
CGTAACAAGG TAG                                                     1513
```

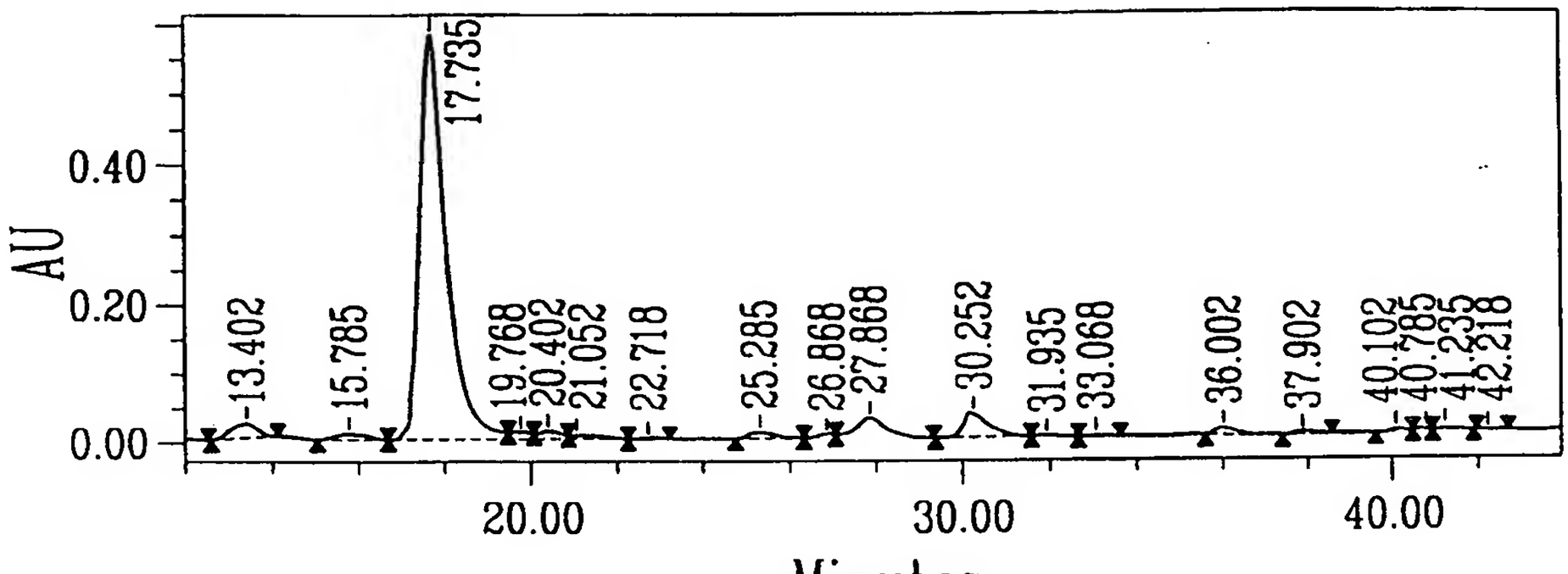
Fig. 8I

```
CGCTGGGCGG CCTGCTTAAC ACnTGCAAGT CGAACGATGA TGCCCAGCTT GCTGGGTGGA    60
TTAGTGGCGA ACGGGTGAGT AACACGTGAG TAACCTGCCC CTGACTCTGG GATAAGCGTT   120
GGAAACGACG TCTAATACTG GATATGATCA CTGGCCGCAT GGTCTGGTGG TGGAAAGATT   180
TTTTGGTTGG GGATGGACTC GCGGCCTATC AGCTTGTTGG TGAGGTAATG GCTCACCAAG   240
GCGACGACGG GTAGCCGGCC TGAGAGGGTG ACCGGCCACA CTGGGACTGA GACACGGCCC   300
AGACTCCTAC GGGAGGCAAC AGTGGGGAAT ATTGCACAAT GGGCGAAAGC CTGATGCAGC   360
AACGCCGCGT GAGGGATGAC GGCCTTCGGG TTGTAAACCT CTTTTAGTAG GGAAGAAGCG   420
AAAGTGACGG TACCTGCAGA AAAAGCACCG GCTAACTACG TGCCAGCAGC CGCGGTAATA   480
CGTAGGGTGC AAGCGTTGTC CGGAATTATT GGGCGTAAAG AGCTCGTAGG CGGTTTGTCG   540
CGTCTGCTGT GAAATCCCGA GGCTCAACCT CGGGCTTGCA GTGGGTACGG GCAGACTAGA   600
GTGCGGTAGG GGAGATTGGA ATTCCTGGTG TAGCGGTGGA ATGCGCAGAT ACCAGGAGGA   660
ACACCGATGG CGAAGGCAGA TCTCTGGGCC GTAACTGACG CTGAGGAGCG AAAGCATGGG   720
GAGCGAACAG GATTAGATAC CCTGGTAGTC CATGCCGTAA ACGTTGGGCG CTAGATGTAG   780
GGACCTTTCC ACGGTTTCTG TGTCGTAGCT AACGCATTAA GCGCCCCGCC TGGGGAGTAC   840
GGCCGCAAGG CTAAAACTCA AAGGAATTGA CGGGGGCCCG CACAAGCGGC GGAGCATGCG   900
GATTAATTCG ATGCAACGCG AAGAACCTTA CCAAGGmTTG ACATACACCG GAAACGGCCA   960
GAGATGGTCG CCCCCTTGTG GTCGGTGTAC AGGTGGTGCA TGGTTGTCGT CAGCTCGTGT  1020
CGTGAGATGT TGGGTTAAGT CCCGCAACGA GCGCAACCCT CGTTCTATGT TGCCAGCGGG  1080
TTATGCCGGG GACTCATAGG AGACTGCCGG GGTCAACTCG GAGGAAGGTG GGGATGACGT  1140
CAAATCATCA TGCCCCTTAT GTCTTGGGCT TCACGCATGC TACAATGGCC GGTACAAAGG  1200
GCTGCGATAC CGTAAGGTGG AGCGAATCCC AAAAAGCCGG TCTCAGTTCG GATTGAGGTC  1260
TGCAACTCGA CCTCATGAAG TCGGAGTCGC TAGTAATCGC AGATCAGCAA CGCTGCGGTG  1320
AATACGTTCC CGGGCCTTGT ACACACCGCC CGTCAAGTCA TGAAAGTCGG TAACACCCGA  1380
AGCCGGTGGC CTAACCCTTG TGGAAGGAGC CGTCGAAGGT GGGATCGGTG ATTAGGACTA  1440
AGTCGTAACA AG                                                      1452
```

Fig. 8J

```
AGAGTTTGAT CATGGCTCAG AACGAACGCT GGCGGCATGC CTAACACATG CAAGTCGAAC    60
GAGATCTTCG GATCTAGTGG CGCACGGGTG CGTAACGCgT gGGAATCTGC cCTTtGGTTC   120
GGAATAaCAG TTGGAAACGA CTGCTAATgA CCGGATGATG ACGTAAGTCC AAAGATTTAT   180
CGCCAGAGGA TkAGCCCGCG TAGGATTAGC tAgTTGGTGT GGTAaGAGCG CACCAAGGcG   240
ACgAtCCTTA GCTGGTCTGA GArGATGAtC AGCCACACTG GGACTGAGAc ACGGcCCAgA   300
CTCCTACGGG AGGCAGCAGT GGGGAATwTT GGACAATGGG CGAAAGCCTG ATCCAGCAAT   360
GCCGCGTGAG TGATGAAGGC CTTAGGGTTG TAAAGCTCTT TTACCCGGGA TGATAATGAC   420
AGTACCGGGA GAATAAGCTC CGGCTAACTC CGTGCCAGCA GCCGCGGTAA TACGGAGGGA   480
GCTAGCGTTG TTCGGAATTA CTGGGCGTAA AGCGCACGTA rGCGGCTTTG TAAGTTAGAG   540
GTGAAAGCCT GGAGCTCAAC TCCAGAATTG CCTTTAAGAC TGCATCGCTT GAATCCAGGA   600
GAGGTGAGTG GAATTCCGAG TGTAGAGGTG AAATTCGTAG ATATTCGGAA GAACACCAGT   660
GGCGAAGGCG GCTCACTGGA CTGGTATTGA CGCTGAGGTG CGAAAGCGTG GGGAGCAAAC   720
AGGATTAGAT ACCCTGGTAG TCCACGCCGT AAACGATGAT AACTAGCTGT CCGGGGACTT   780
GGTCTTTGGG TGGCGCAGCT AACGCATTAA GTTATCCGCC TGGGGAGTAC GGCCGCAAGG   840
TTAAAACTCA AATGAATTGA CGGGGGCCTG CACAAGCGGT GGAGCATGTG GTTTAATTCG   900
AAGCAACGCG CAGAACCTTA CCAGCGTTTG ACATGTCCGG ACGATTTCTG GAGACAGATC   960
TCTTCCCTTC GGGGACTGGA ACACAGGTGC TGCATGGCTG TCGTCAGCTC GTGTCGTGAG  1020
ATGTTGGGTT AAGTCCCGCA ACGAGCGCAA CCCTCGCCTT TAGTTACCAT CATTTAGTTG  1080
GGGACTCTAA AGGAACCGCC GGTGATAAGC CGGAGGAAGG TGGGGATGAC GTCAAGTCCT  1140
CATGGCCCTT ACGCGCTGGG CTACACACGT GCTACAATGG CGGTGACAGT GGGCAGCAAA  1200
CTCGCGAGAG TGCGCTAATC TCCAAAAGCC GTCTCAGTTC GGATTGTTCT CTGCAACTCG  1260
AGAGCATGAA GGCGGAATCG CTAGTAATCG CGGATCAGCA TGCCGCGGTG AATACGTTCC  1320
CAGGCCTTGT ACACACCGCC CGTCACACCA TGGGAGTTGG GTTCACCCGA AGGCGTTGCG  1380
CTAACTCAGC AATGAGAGGC AGGCGACCAC GGTGGGCTTA GCGACTGGGG TGAAGTCGTA  1440
ACAAGGTAAC                                                         1450
```

FIG. 8K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0.20
0.00
13.402
15.785
17.735
19.768
20.402
21.052
22.718
25.285
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27.868
30.252
31.935
33.068
36.002
37.902
40.102
40.785
41.235
42.218
20.00
30.00
40.00
Minutes

FIG 9A

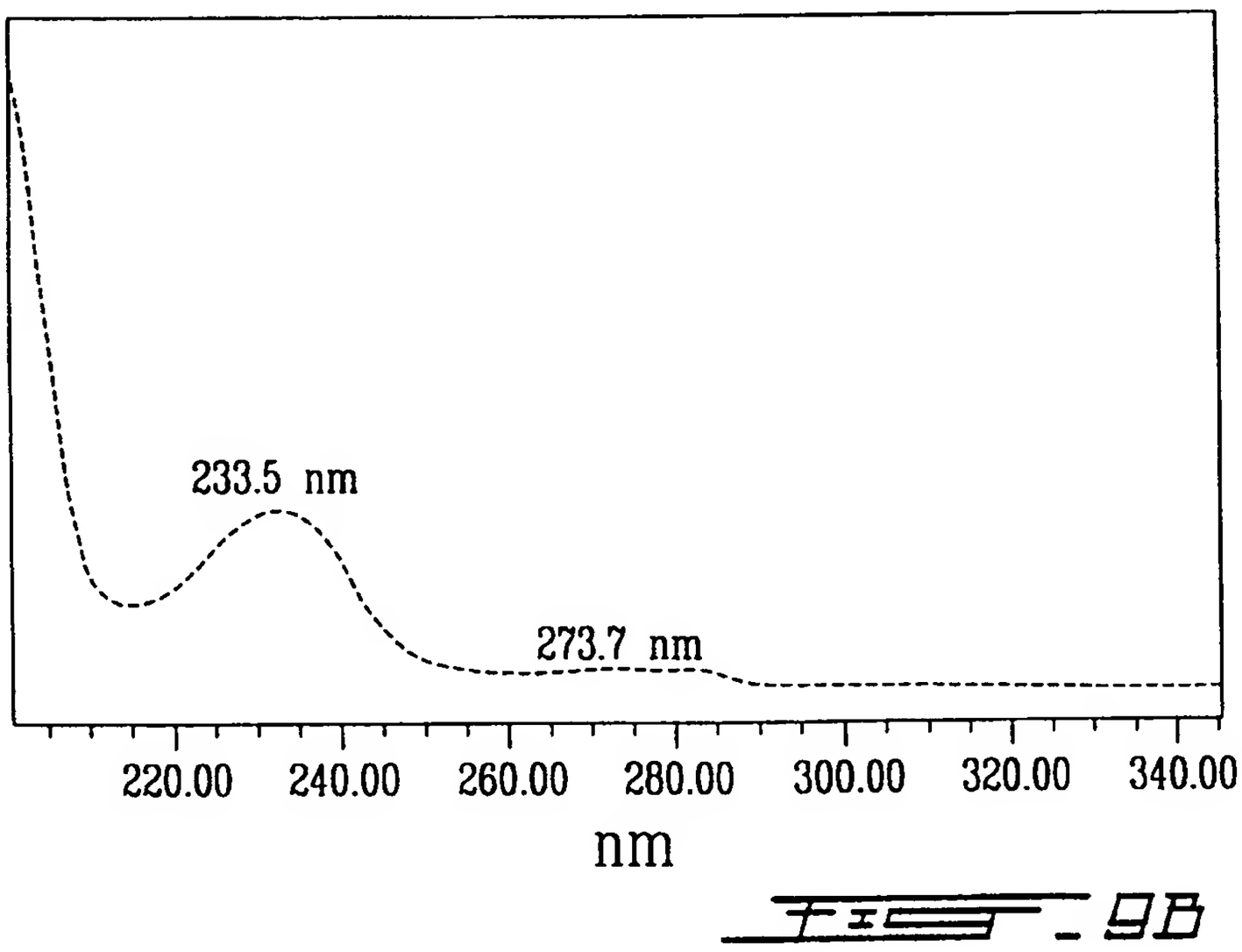
233.5 nm
273.7 nm
220.00
240.00
260.00
280.00
300.00
320.00
340.00
nm

FIG 9B

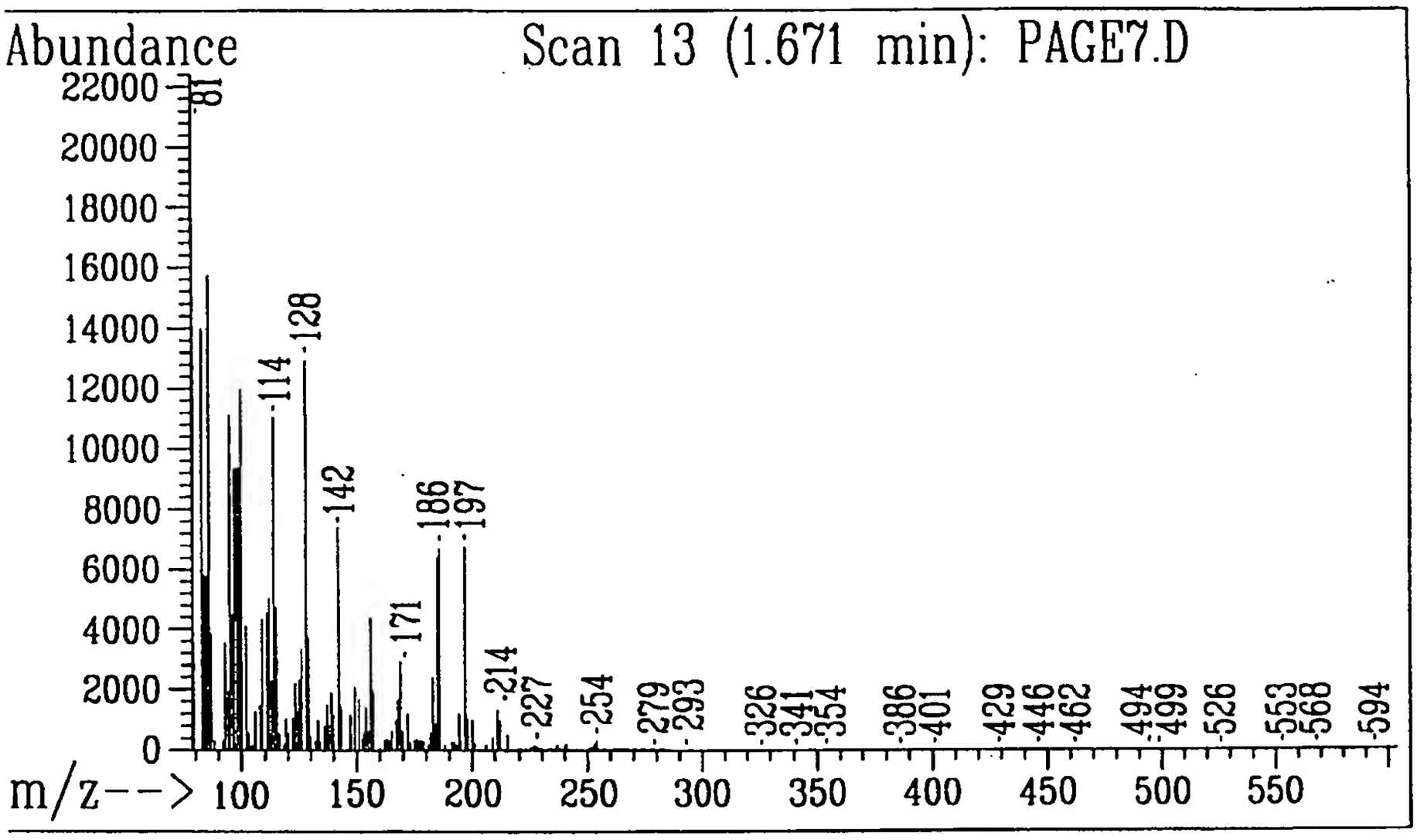

FIG_10A

m/z	Assignment
447	$(T-BzOH)^+$
386	$(T-H_2O-BzOH-AcOH)^+$
326	$(T-BzOH-2AcOH-H_2O)^+$
308	$(T-BzOH-2AcOH-2H_2O)^+$

FIG_10B

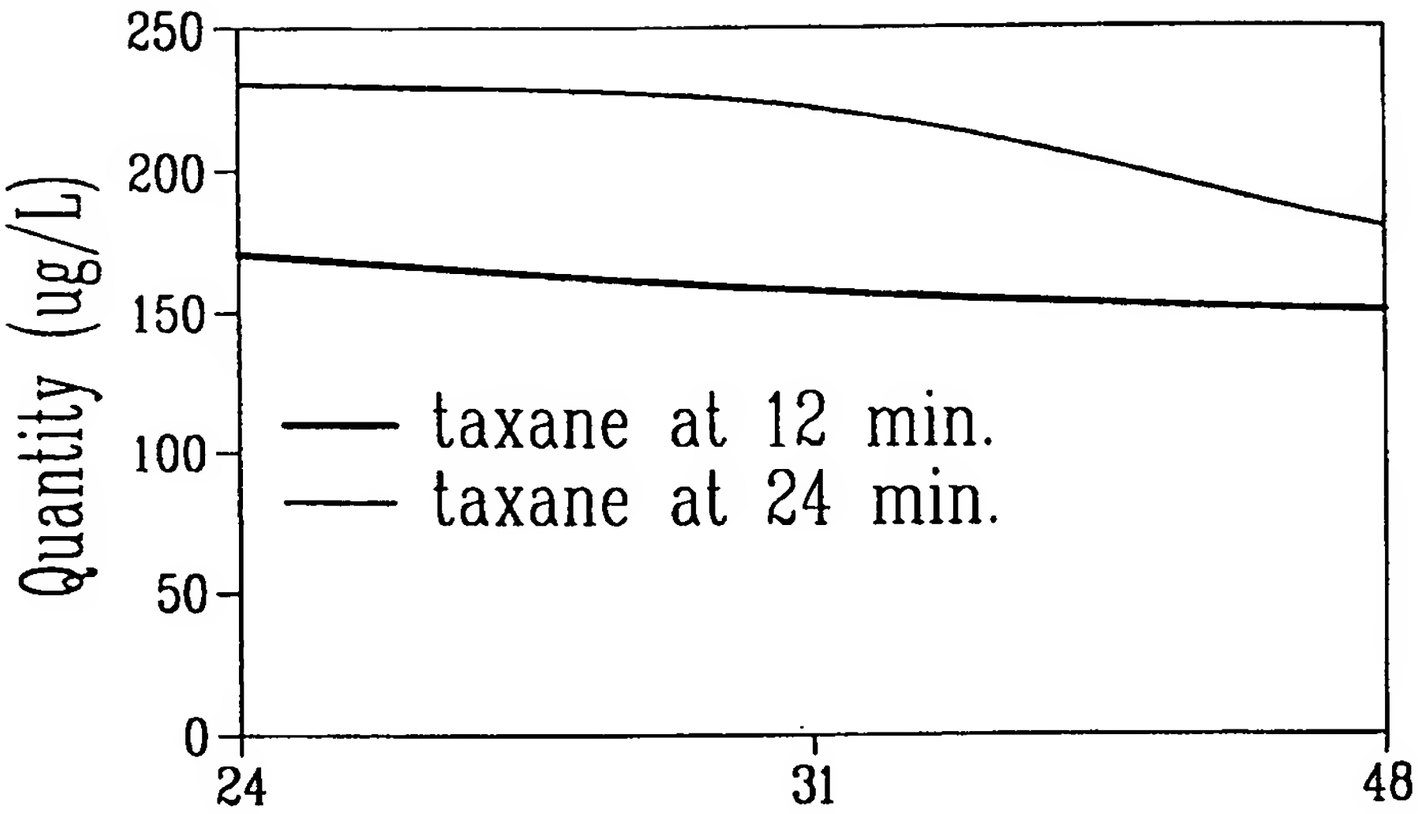

FIG - 11A

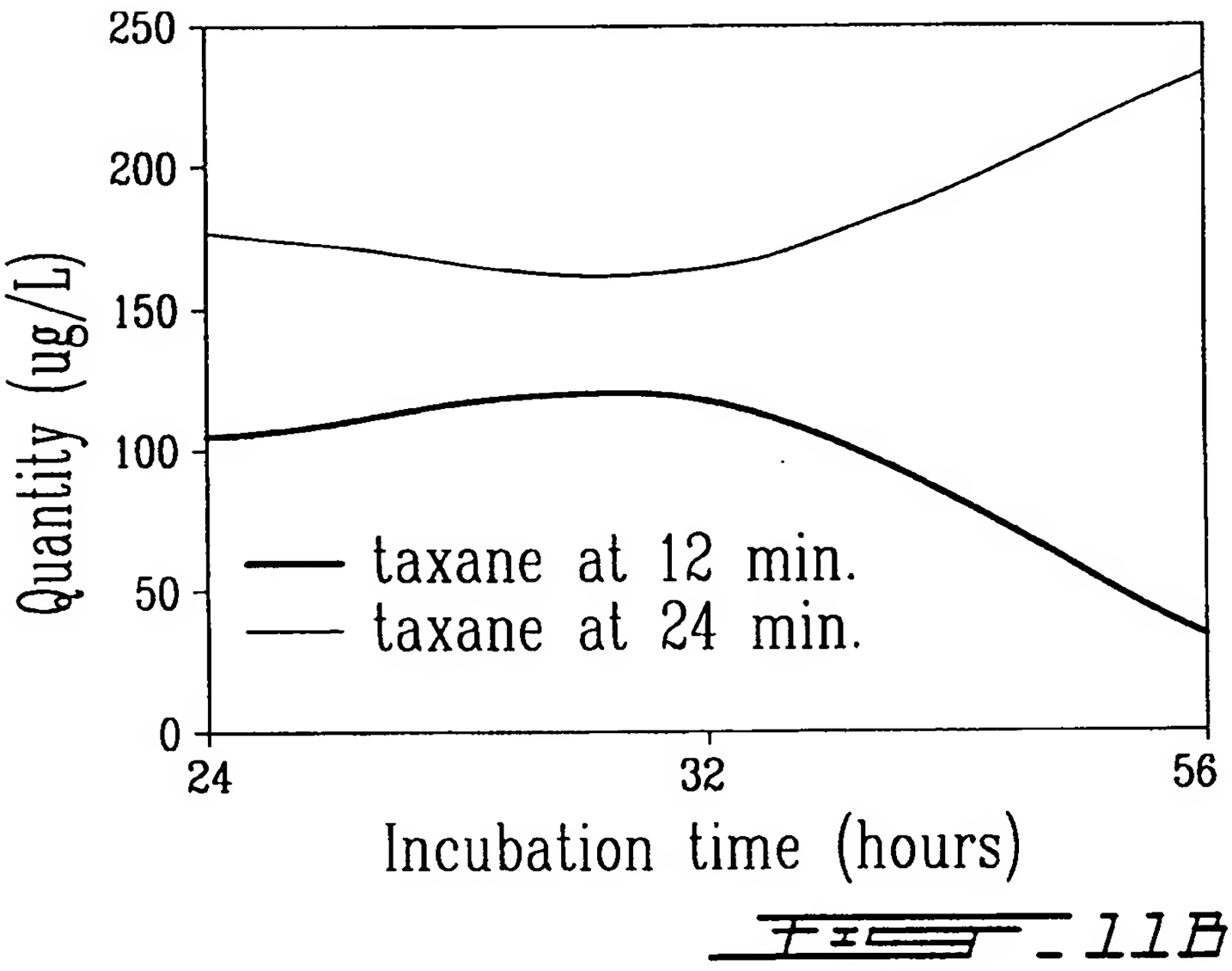

FIG - 11B

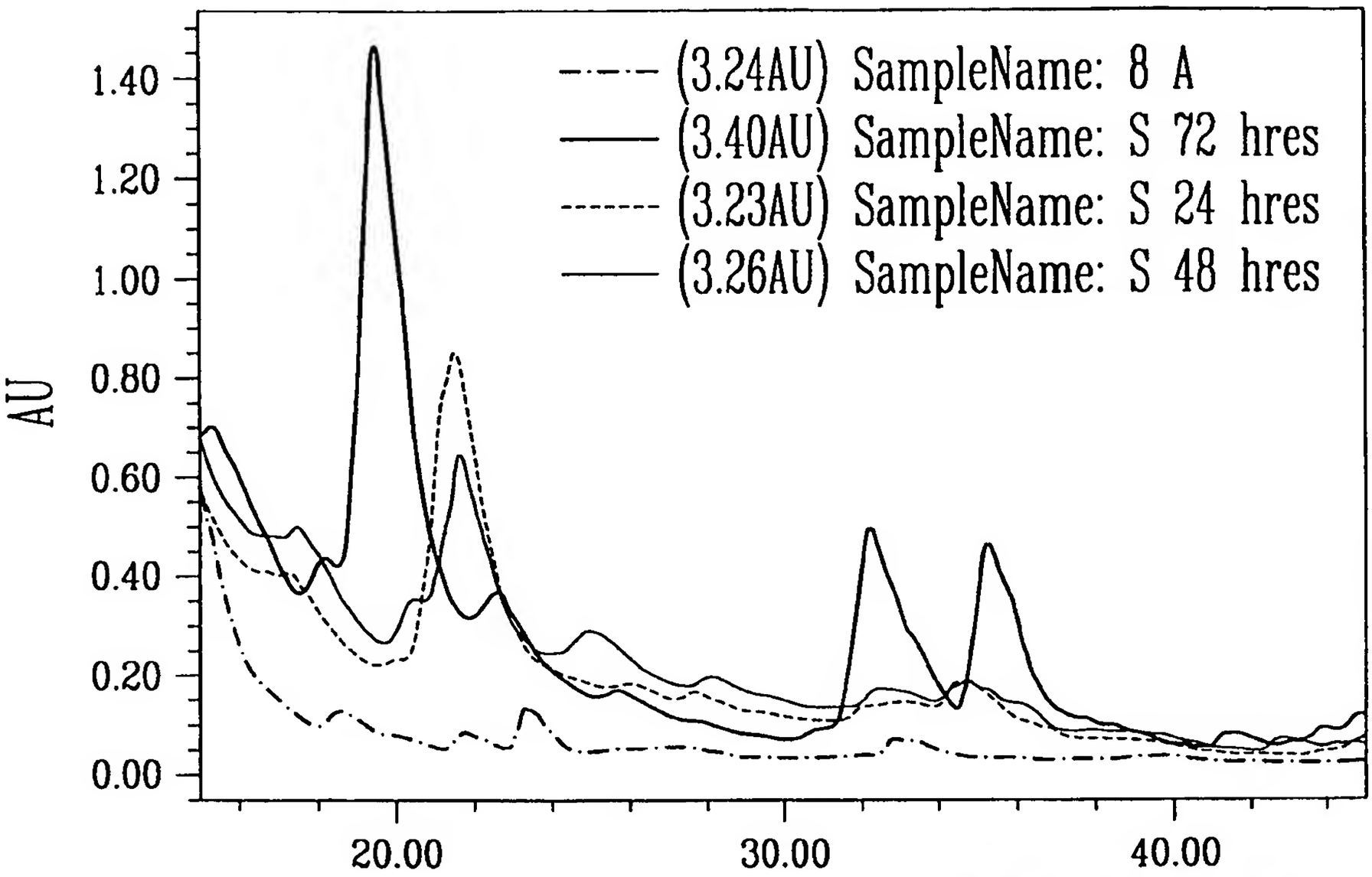
(3.24AU) SampleName: 8 A
(3.40AU) SampleName: S 72 hres
(3.23AU) SampleName: S 24 hres
(3.26AU) SampleName: S 48 hres
AU
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
20.00
30.00
40.00
Minutes

Fig 12

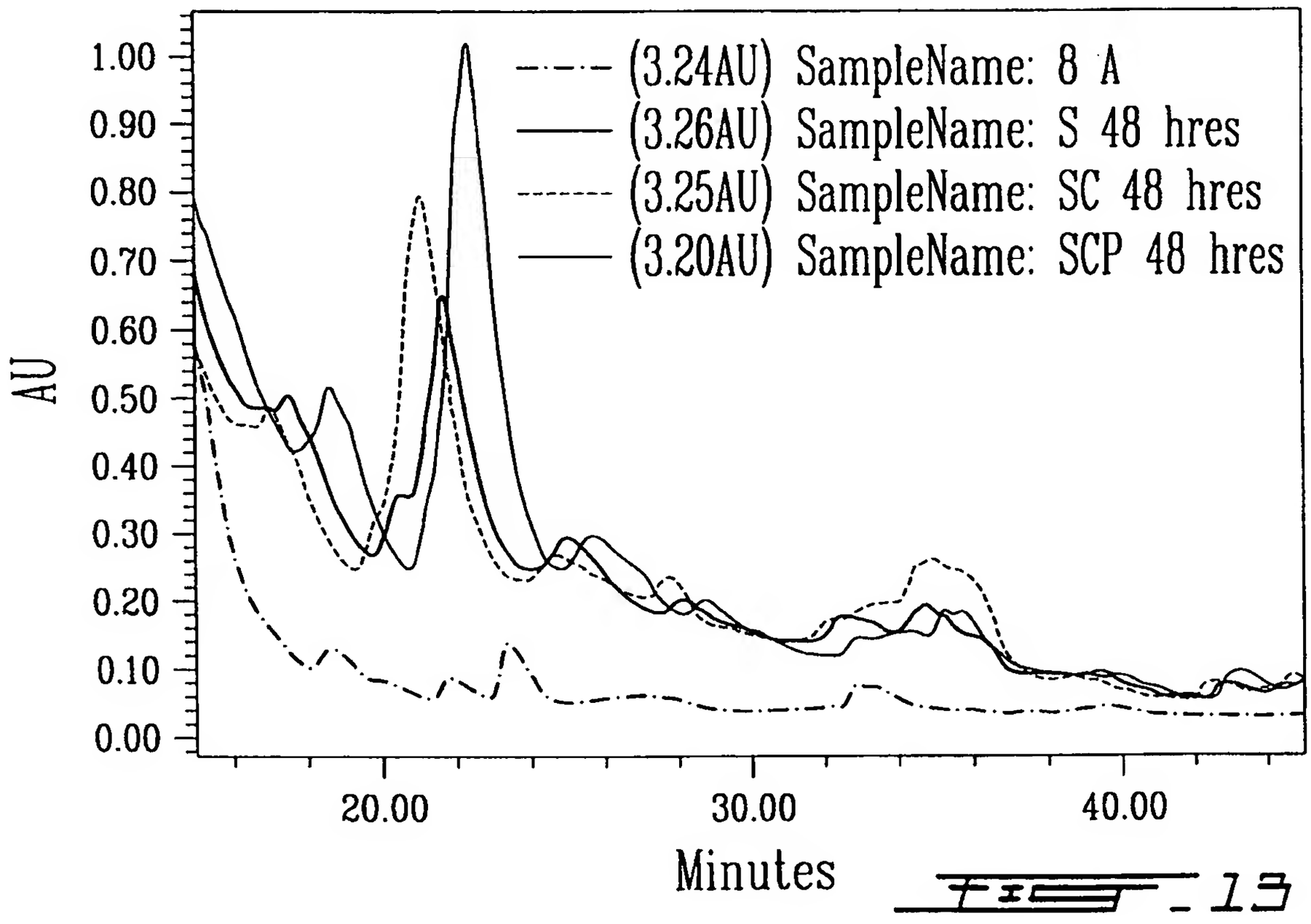
(3.24AU) SampleName: 8 A
(3.26AU) SampleName: S 48 hres
(3.25AU) SampleName: SC 48 hres
(3.20AU) SampleName: SCP 48 hres
AU
1.00
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
20.00
30.00
40.00
Minutes

Fig 13

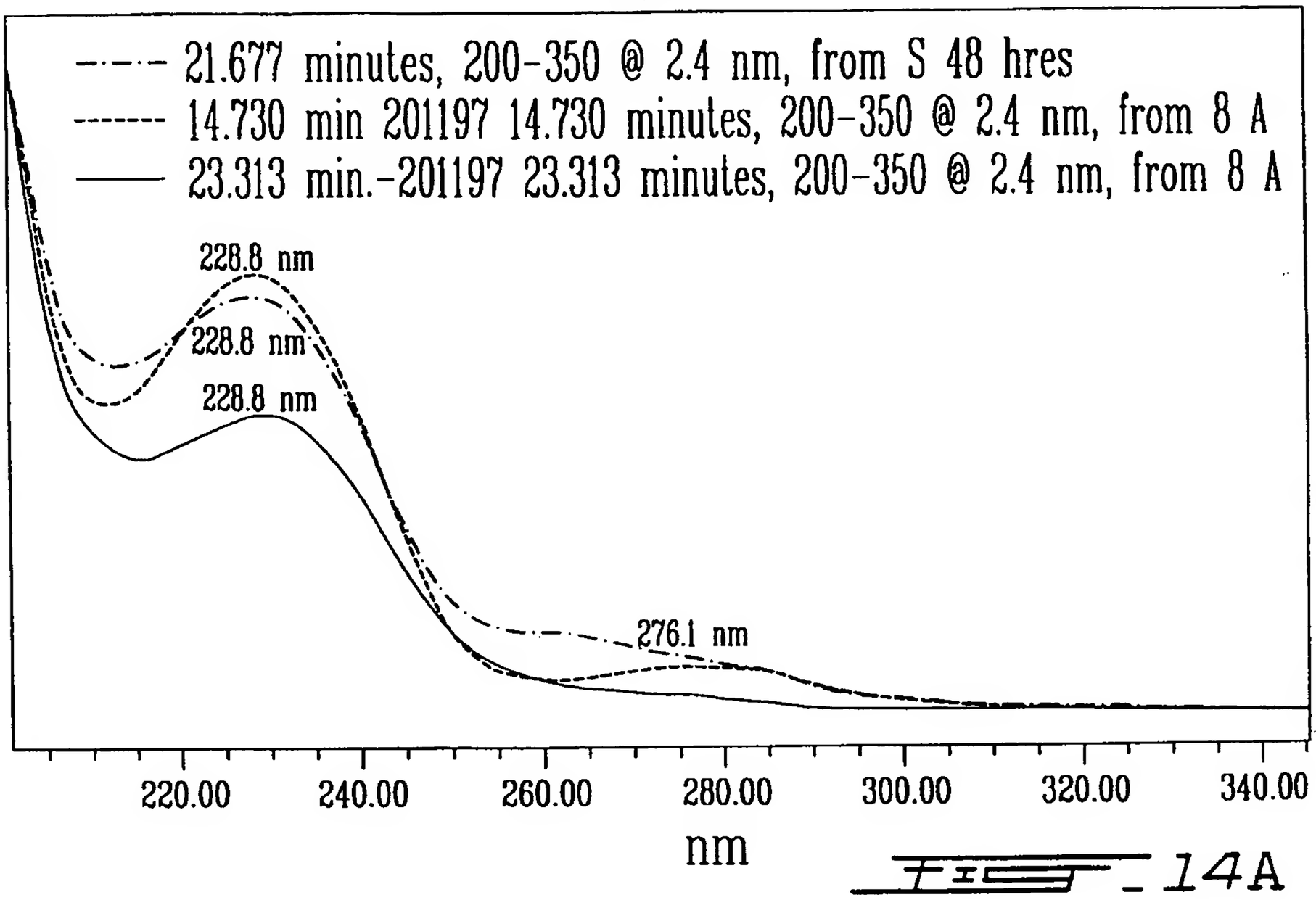

Fig 14A

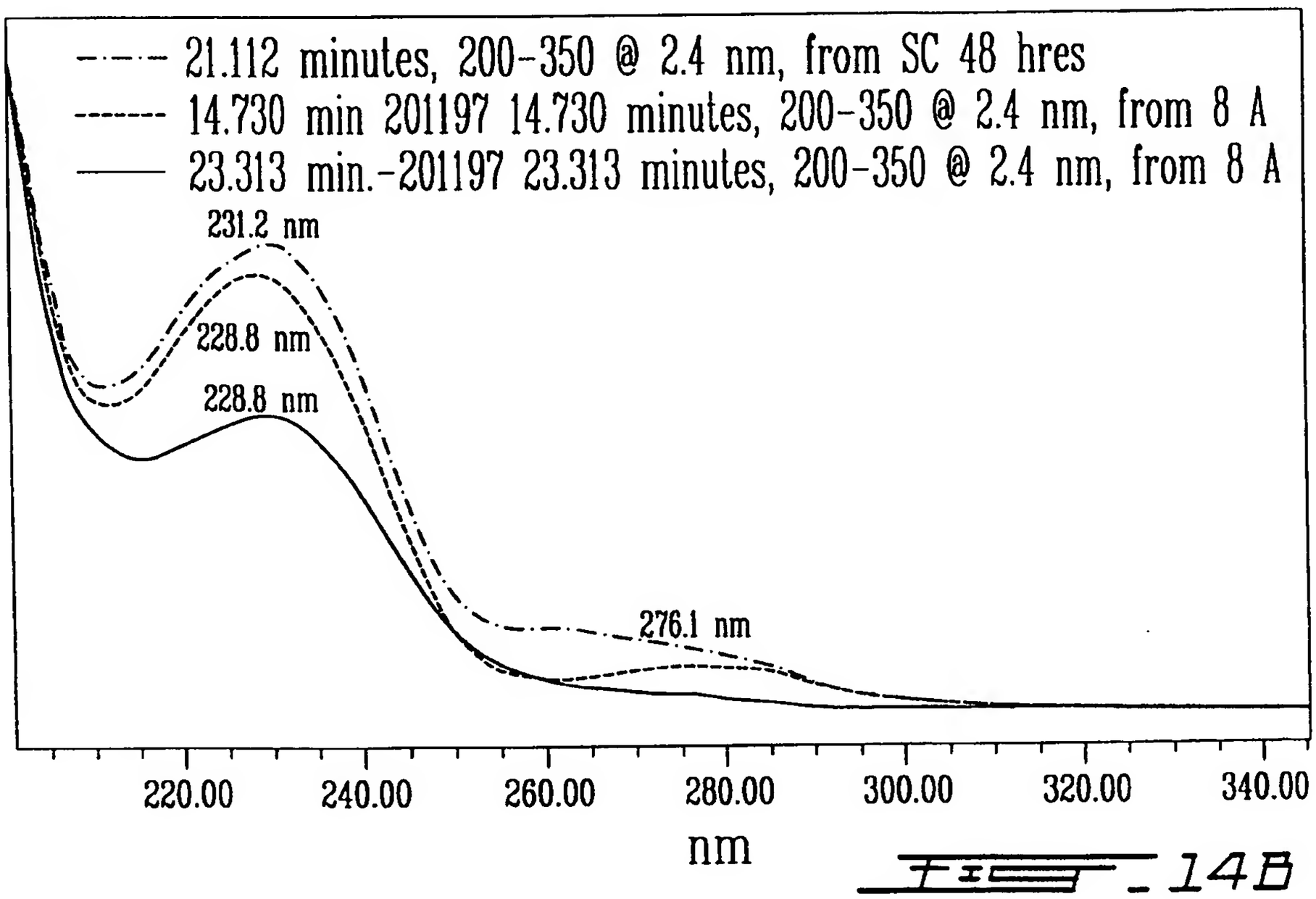

Fig 14B

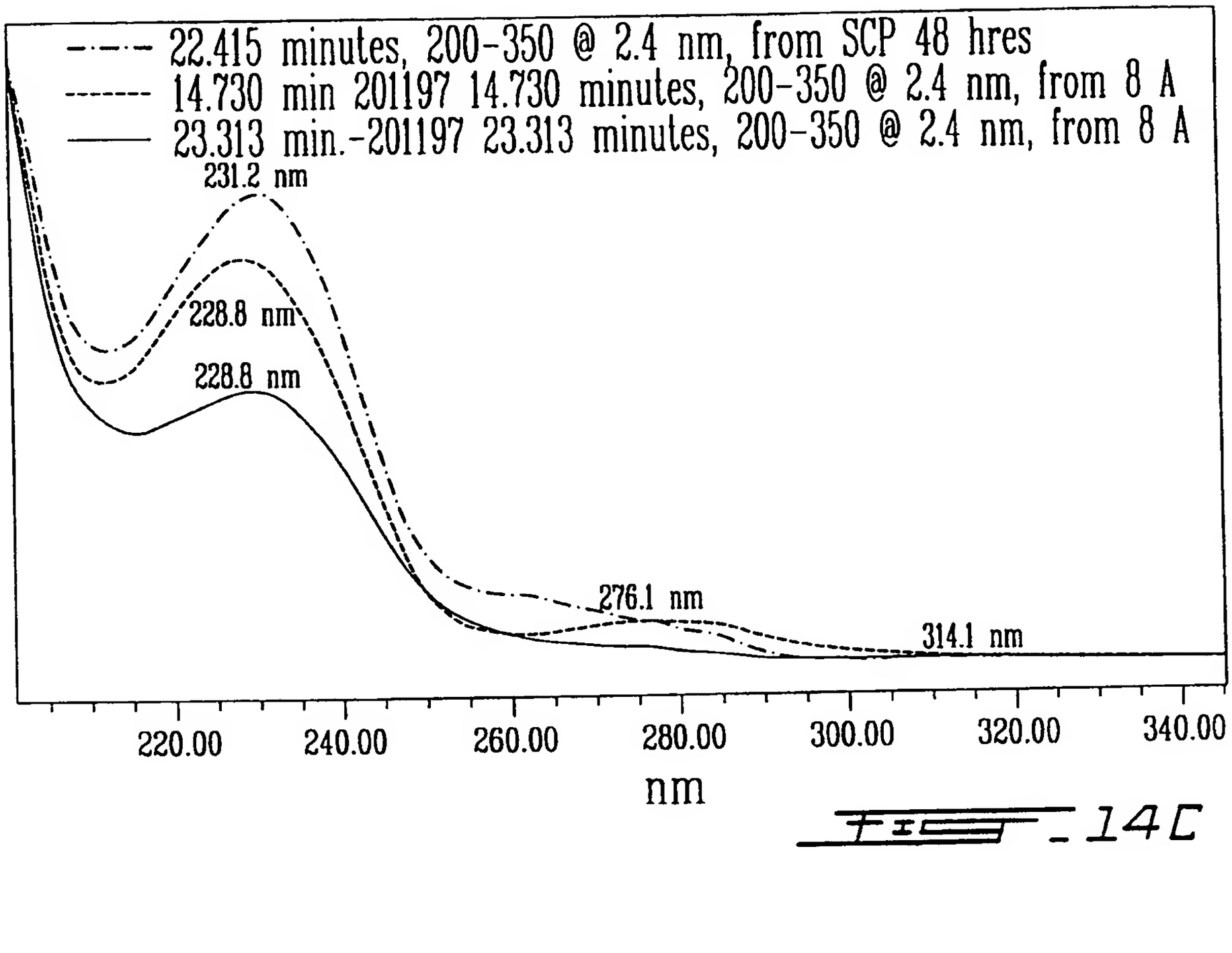
22.415 minutes, 200-350 @ 2.4 nm, from SCP 48 hres
14.730 min 201197 14.730 minutes, 200-350 @ 2.4 nm, from 8 A
23.313 min.-201197 23.313 minutes, 200-350 @ 2.4 nm, from 8 A
231.2 nm
228.8 nm
228.8 nm
276.1 nm
314.1 nm
220.00
240.00
260.00
280.00
300.00
320.00
340.00
nm

Fig 14C

SEQUENCE LISTING

<110> BCM Développement Inc.
PAGÉ, Michel
LANDRY, Nathalie
BOISSINOT, Maurice
HÉLIE, Marie-Claude
HARVEY, Mario
GAGNÉ, Martin

<120> BACTERIAL MASS PRODUCTION OF TAXANES AND PACLITAXEL

<130> 12637-2PCT FC/ld

<150> US 08/995,960

<151> 1997-12-22

<160> 13

<170> FastSEQ for Windows Version 3.0

<210> 1
<211> 1556
<212> DNA
<213> Sphingomonas taxi

<400> 1

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<210> 2
<211> 1512
<212> DNA
<213> Bacillus cereus ssp. taxi

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gtaacaaggt aa 1512

<210> 3
<211> 1515
<212> DNA
<213> Bacillus megaterium ssp. taxi

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<210> 4
<211> 1451
<212> DNA
<213> Pantoea sp. BCM 1

<400> 4
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<210> 5
<211> 1513
<212> DNA
<213> Bacillus cereus ssp. BCM 4

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<210> 6
<211> 1512
<212> DNA
<213> Bacillus subtilis ssp. taxi

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<210> 7
<211> 1458
<212> DNA
<213> Pantoea sp. BCM 2

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<210> 8
<211> 1451
<212> DNA
<213> Pantoea sp. BCM 3

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cgcataacgt cgcaagacca aagaggggga ccttcgggcc tctcactatc ggatgaaccc 180
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<210> 9
<211> 1513
<212> DNA
<213> Bacillus megaterium ssp. BCM 9

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aacctgcctg taagactggg ataacttcgg gaaaccgaag ctaataccgg ataggatctt 180
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INTERNATIONAL SEARCH REPORT

Int ional Application No
PCT/CA 98/01150

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12P17/02 C12N3/00 C12N1/00 C12N15/01 C12N1/20
C12P7/62 C12P7/26 //(C12P17/02,C12R1:07),(C12P17/02,
C12R1:125)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12P C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	WO 96 34972 A (BCM DEVELOPMENT INC.) 7 November 1996 cited in the application see the whole document ---	1-57
X	EP 0 668 360 A (BRISTOL-MYERS SQUIBB COMPANY) 23 August 1995 see page 6 - page 7; claims 1-21 --- -/--	1-57

[X] Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search	Date of mailing of the international search report
27 May 1999	10/06/1999

Name and mailing address of the ISA	Authorized officer
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X,L	CHEMICAL ABSTRACTS, vol. 130, 1999 Columbus, Ohio, US; abstract no. 174870, JAZIRI, M. ET AL: "A new concept for the isolation of bioactive taxoids" XP002103986	1-40
Y	see abstract & CURR. TOP. PHYTOCHEM. (1997), 1, 67-76 CODEN: CTPHFK,	41-57
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Y	see page 1 - page 6; claims 1-9	1-5,7-57
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Inte ional Application No
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